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(54) Glycosyl-Phosphatidylinositol-Specific Phospholipase D.

(57) The present invention relates to the protein glycosyl phosphatidylinositol-specificphospholipase D (GPI-PLD) in a substantially pure form, an polynucleotide coding for GPI-PLD, vectors containing the isolated polynucleotide coding for GPI-PLD, and cells transformed by a vector containing the polynucleotide coding for GPI-PLD. Also described is a method for producing a protein which can be secreted from a eukaryotic cell comprising co-transfecting a eukaryotic cell with a gene encoding a glycosyl phosphatidylinositol-anchored protein with glycosyl phospatidylinositol-specific phospholipase D.

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The present invention lies in the fields of protein, recombinant DNA and genetic engineering.

Recent studies have revealed that a growing number of cell surface proteins are attached to the membrane by covalent linkage to a glycosylphosphatidylinositol (GPI) anchor. The physiological role played by this new class of membrane anchor is unknown, but one possibility is that it facilitates the release of molecules by specific phospholipases in vivo.

Several mammalian phospholipase activities which seem to be capable of removing the GPI anchors from proteins have been reported. These were originally attributed to the action of a phosphatidylinositol (PI)-specific phospholipase C since enzymes of this specificity are widely distributed in mammalian tissues. However, the physiological significance of such a process remained in question because almost all of the mammalian PI-specific phospholipase C's are believed to be intracellular in location whereas the GPI-anchored proteins are found on the cell surface. Subsequently, it was shown that inhibition of placental PI-specific phospholipase C activity does not affect GPI-anchor degrading activity, indicating that other enzymes are responsible for the release of GPI-anchored proteins. It was therefore suggested that this activity was due to a novel phospholipase D with specificity for the GPI-anchor. Recently, several groups have reported the presence of high levels of a GPI-specific phospholipase D (GPI-PLD) in mammalian plasma and serum [Low, M. G., and Prasad, A. R. S. (1988) Proc. Natl. Acad. Sci. USA, 85, 980-984; Davitz, M. A., Hereld, D., Shak, S., Krakow, J., Englund, P. L., and Nussenzweig, V. (1987) Science, 238, 81-84; Cardoso de Almeida, M. L., Turner, M. J., Stambuk, B. B. and Schenkman, S. (1988) Biochem. Biophys. Res. Commun., 150, 476-482)]. Because of its extracellular location and specificity for GPI, this enzyme may be responsible for releasing GPI-anchored proteins from cell surfaces in vivo.

The present invention relates to the protein glycosyl phosphatidylinositol-specificphospholipase D (GPI-PLD) or biologically active fragments thereof substantially free from other proteins, polynucleotides encoding GPI-PLD or biologically active fragments thereof, vectors containing a polynucleotide encoding GPI-PLD or a biologically active fragment thereof, and cells transformed by such a vector.

In another embodiment, the present invention also relates to mutations of GPI-PLD or of biologically active fragments thereof which substantially retain the biological activity of natural GPI-PLD, polynucleotides coding for these mutants, vectors containing these polynucleotides, and cells transformed by such a vector.

The present invention also relates to a process for producing GPI-PLD, a biologically active fragment thereof or a mutant of GPI-PLD or a fragment thereof, comprising culturing a host containing a recombinant vector which codes for such a GPI-PLD active compound under appropriate conditions of growth so that said compound is expressed and isolating said compound.

Another embodiment of the present invention is a method for producing a secretable protein from a eukaryotic cell comprising co-transfecting a eukaryotic cell with a gene encoding a glycosyl phosphatidylinositol-anchored protein with glycosyl phosphatidylinositol-specific phospholipase D.

Additionally, the present invention relates to a process for cleaving proteins which are anchored to a cell by means of a glycosyl phosphatidyl inositol anchor comprising administering to the cell culture in which the cell is growing glycosyl phosphatidylinositol-specific phospholipase D in combination with a suitable detergent.

Finally, the present invention relates to antibodies specific to GPI-PLD substantially free from other proteins.

Brief Description of the Drawings

Fig. 1. A Model of a GPI anchor structure. The COOH-terminal amino acid of the protein is linked to an ethanolamine residue which in turn is linked via a phosphodiester bond to a complex glycan moiety. The site of GPI-PLD hydrolysis is marked.

Fig. 2. SDS-PAGE of Samples Purified from Hydroxyapatite and Zn-chelate Matrix Chromatographies. Samples (1-3 μg) were run on 10% polyacrylamide gels under reducing conditions and were visualized by Coomassie Blue staining. Protein standards (prestained) were from Bethesda Research Labs. Lane 1: hydroxyapatite flow-through pool (~3 μg); lane 2: Zn-chelate pool 1 (~1 μg); lane 3: Zn-chelate pool 2 (~3 μg).

Fig. 3. SDS-PAGE of Samples Purified by the Immunoaffinity Chromatography Procedure. Samples were run on 8.5% acrylamide gels under reducing conditions and were visualized by Coomassie Blue staining. Lane 1: immunoaffinity-eluate, ~10 μg; late 2: lectin Sepharose-eluate, ~5.0 μg; lane 3: Mono Q-FPLC pool ~2.5 μg.

Fig. 4. Restriction Map and sequencing strategy of the spliced inserts from Clones pBJ1549 and pBJ1644. The bovine liver GPI-PLD cDNA inserts from the two lambda gt11 clones were subcloned into pGEM4Z (Promega Biotec, Madison, WI) and both strands sequenced using

Sequenase enzyme (U.S. Biochemical Corp., Cleveland, OH). Arrows with closed and open circles represent sequences determined from SP6/T7 promoter primers of smaller subclones and sequences determined from synthetic oligonucleotide primers, respectively. The positions of the translation start and stop codons are marked. Clones pBJ1549 and pBJ1644 extended from nucleotides 1-1577 and 1438-2578, respectively. A, AccI; b, BamHI; H, HindIII; K, KpnI; N, NcoI; P,PstI; S, SacI; V, PvuII.

Fig. 5. DNA sequence and deduced amino acid sequence of bovine liver GPI-PLD. The arrow marks the N-terminus of the mature protein. Regions showing sequence similarity to metal ion binding domains of integrin α subunits are underlined.

Fig. 6. Western Blot Analysis of Transfected COS Cell Media and Lysates. The complete 2.6 kb cloned cDNA was ligated into the HindIII/SmaI site of pBC12B1 and the recombinant plasmid, pBJ1682, introduced into COS-7 cells utilizing standard DEAE-dextran mediated methods. COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum for the first 24 hours then switched to serum-free DMEM containing 1% Nutridoma (Boehringer Mannheim, Indianapolis, IN) to rid of endogenous PLD. Media was collected 48 hours after switching to serum-free media, centrifuged to pellet any suspended cells and concentrated 15-fold using Centricon 10 (Amicon, Danvers, MA). Cell lysates were prepared at a concentration of 5×10^7 cell-equivalents per ml in 0.5% Nonidet P-40 in PBS containing aprotinin (30 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), and phenylmethyl sulfonyl flouride (1 mM). Lysates were centrifuged at 13,000 g for 20 min at 4°C and the supernatant collected. After gel electrophoresis and blotting to nitrocellulose, proteins were detected using a pool of five monoclonal antibodies (1 μg/ml each) against bovine serum GPI-PLD and alkalinephosphatase conjugated goat anti-mouse IgG (Jackson Immuno-Research, West Grove, PA). Lane 1, lysate of mock-transfected cells; Lane 2, medium from mock-transfected cells; Lane 3, equal amounts of lysate from pBJ1682-transfected cells and medium from mock-transfected cells; Lane 4, equal amounts of medium from pBJ1682-transfected cells and lysate from mock-transfected cells; Lane 5, 50 ng of purified serum GPI-PLD; Lane 6,200 ng of purified serum GPI-PLD mixed with mock-transfected cell medium.

Fig. 7. Demonstration of GPI-PLD Activity in Transfected COS Cells by Hydrolysis of ^{3}H-labelled VSG. Mock-transfected or pBJ1682-transfected COS cells were switched to serum-free media 24 hours after transfection as described for Fig. 6. (A) Time dependence of phospholipase activity. At various time points after switching to serum-free medium, aliquots (10 μl each) were withdrawn from the medium and assayed for phospholipase activity. One unit of activity was defined as the amount of enzyme hydrolyzing 1% of the [^{3}H]myristate-labelled VSG per min. Δ.....Δ indicates the activity in DNA-transfected cells; o.....o indicates the activity in mock-transfected cells. (B) Comparison of phospholipase activity in the medium and cell lysate. After cells were grown in serum-free medium for 44 hours, 10 μl of medium was withdrawn and assayed for phospholipase activity. Cells lysates were prepared as described in Fig. 6 and assayed at the same time.

Fig. 8. Product Analysis of Hydrolyzed VSG by Thin-layer Chromatography. Samples (50 μl) of pBJ1682- and mock-transfected COS cell media and GPI-PLD purified from serum were incubated at 37° C for 30 min with 100 μl of VSG cocktail consisting of 40 μM Tris-maleate, pH 7.0, 0.2% NP-40, and 3×10^4 cpm ^{3}H-labelled VSG. The reaction were terminated with the addition of 0.5 ml butanol and spiked with 25 μg each of dimyristoyl phosphatidic acid (DMPA) and dimyristoyl glycerol (DMG). After phase separation by centrifugation, 0.35 ml of the upper butanol phases were evaporated to dryness and the reaction products resuspended in 20 μl of $CHCl_3$:MeOH (1:1, v/v) and spotted onto a silica gel 60F254 plate (Merck), along with DMPA and DMG standards. The plate was run in a solvent system consisting of $CHCl_3$:pyridine:70% formic acid (50:30:7, v/v/v). After development for a distance of 10cm, the plate was air dried overnight and the standards visualized with iodine. Zones of 0.5 cm were scraped, eluted with 150 μl $CHCl_3$:MeOH:butanol (1:1:1, v/v/v), and counted in a scintillation fluor. O----O, mock-transfected cell media; □ ---- □ , pBJ1682-transfected cell media; •----•, purified serum GPI-PLD.

Fig. 9. Nucleotide sequence and deduced amino acid sequence of the human liver glycosyl phosphatidylinositol specific-phospholipase D.

Fig. 10. The Alignment of amino acid sequence of the human and bovine liver GPI-PLD mature protein.

Fig 11. Nucleotide sequence and deduced amino acid sequence of the human pancreatic glycosyl phosphatidylinositol specific-phospholipase D.

Fig. 12. SDS PAGE showing the expression of ELAM-1-GPI (Column A) and ELAM-1-2-GPI (Column B) with PLD and without PLD.

Additionally, in the present application the following abbreviations are used: GPI, glycosyl-phosphatidylinositol; PLD, phospholipase D; PI, phosphatidylinositol; VSG, variant surface glycoprotein; CHAPS, (3-[(3-cholamidopropyl) dimethyl- ammonio] 1-propanesulfonate; PEG, polyethylene glycol; BSA, bovine serum albumin; EGTA, ethylenebis(oxyethylenenitrilo) tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HP(or FP)LC, high performance (or fast protein) liquid chromatography; PTH, phenylthiohydantoin; ELISA, enzyme-linked immunosorbent assay; HRP, horse radish peroxidase.

The teachings of all of the references cited herein are hereby incorporated by reference.

Detailed Description of the Invention

The present invention relates to glycosyl phosphatidylinositol-specific phospholipase D (GPI-PLD) or biologically active fragments thereof substantially free from other proteins. This enzyme selectively hydrolyzes the inositol-phosphate linkage of glycosyl phosphatidylinositol (GPI)-anchored proteins, GPI lipids and related molecules. See Figure 1.

The DNA sequence and deduced amino acid sequence of bovine liver GPI-PLD is shown in Figure 5; the DNA sequence and deduced amino acid sequence of human liver GPI-PLD is shown in Figure 9; and Figure 11 shows the DNA sequence and the deduced amino acid sequence of human pancreatic GPI-PLD. Using conventional methods of recombinant DNA technology, (see for example Maniatis et al., "Molecular Cloning - A Laboratory Manual". Cold Spring Harbor Laboratory. 1989) expression vectors encoding for recombinant GPI-PLD can be constructed. Upon introduction of these expression vectors into a prokaryotic and eukaryotic host, recombinant GPI-PLD is synthesized.

The invention also relates to a polynucleotide, either double or single stranded, coding for a GPI-PLD protein or a biologically active fragment thereof. The nucleotide sequences which are coding for bovine liver GPI-PLD, human liver GPI-PLD and human pancreatic GPI-PLD are shown in Figures 5, 9 and 11, respectively.

The invention therefore relates to these nucleotide sequences or to homologous or degenerate sequences thereof, that means to nucleotide sequences having the same function, but originating from a different species (e.g. from human) or to nucleotide sequences being degenerate in the genetic code. The polynucleotide can be obtained from natural sources or be prepared synthetically by methods known to the person skilled in the art.

Moreover, the invention relates to replicable microbial vectors containing a polynucleotide with a sequence which codes for a polypeptide having GPI-PLD activity, to host organisms transformed with such a replicable microbial vector, which host is capable of expressing the amino acid sequence encoded by said polynucleotide.

A wide variety of host/cloning vehicle combinations may be employed in cloning the double-stranded DNA. For example, useful cloning vehicles may consist of segments of chromosomal, nonchromosomal or synthetic DNA sequences, such as various known bacterial plasmids, e.g. plasmids from E. coli such as pBR322, phage DNA, and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA or other expression control sequences or yeast plasmids. Useful hosts include microorganisms, mammalian cells, plant cells and the like. Among them microorganisms and mammalian cells are preferably employed. As preferable microorganisms, there may be mentioned yeast and bacteria such as Escherichia coli or Actinomyces. Among mammalian hosts CHO cells are preferred.

A cloning vehicle or vector containing the foreign polynucleotide is employed to transform a host so as to permit that host to express the protein or portion thereof for which the polynucleotide codes. The selection of an appropriate host is also controlled by a number of factors recognized inthe art. These include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, biosafety and costs. A suitable expression vector for use in the present invention is the eukaryotic expression plasmid pBC12BI (Cullen- (1987), Methods in Enzymology 152, 684-704). Other suitable cloning or expression vectors are disclosed in the examples or are known in the art.

The invention also relates to mutations of GPI-PLD or biologically active fragments thereof which substantially retain the biological activity of natural GPI-PLD. The invention also relates to a polynucleotide

coding for the mutant GPI-PLD or a biologically active fragment thereof. Furthermore, the invention relates to vectors containing the isolated polynucleotide coding for the mutant GPI-PLD or biologically active fragments thereof. These mutants can be produced by known methods such as site-specific mutagenesis of the DNA sequence and the mutant DNA construct inserted into an expression vector and the expression vector introduced into a suitable prokaryotic or eukaryotic host to produce a mutated form of GPI-PLD. A mutated form of GPI-PLD can also be produced by means of enzymatic cleavage of the GPI-PLD protein and solid phase synthesis. The mutated form of the protein can then be assayed for its ability to exhibit PLD activity by assays herein described.

According to the present invention, GPI-PLD is purified and characterized by the method described in Example 1. The procedure, which was developed and can be used for the purification of GPI-PLD, is discussed below and describes the identification of the active enzyme compounds obtained during the individual purification steps.

In this procedure, the bulk of serum albumin and some other contaminating proteins were removed by PEG precipitation. The supernatant was then chromatographed on Q Sepharose (anion exchange) followed by S-300 gel filtration chromatography. GPI-PLD activity eluted in the broad second protein peak with a molecular weight of ≧250 kDa. This broad elution of activity suggests that GPI-PLD in serum may form a complex with other serum proteins.

GPI-PLD was further purified by wheat germ lectin-Sepharose and hydroxyapatite chromatography. At this stage, GPI-PLD was about 10% pure as judged by SDS-PAGE using procedures described by Lemmli, U.K. (1970), Nature, 227, 680-685. The final stages of purification consisted of Zn-chelate chromatography, Mono Q-HPLC and Superose 12-HPLC. When hydroxyapatite-purified material was chromatographed on Zn-chelate, two GPI-PLD activity peaks were observed. The first activity peak (pool 1) eluted in the wash fractions, separated from the majority of contaminating proteins and had the higher specific activity. This pool contained a major protein band on SDS-PAGE with an apparent molecular weight of ~100 kDa in addition to other minor protein bands. The second activity peak (pool 2) eluted with 10 mM histidine and contained two major protein bands with molecular weights of ~100 and ~180 kDa and several minor components on SDS-PAGE.

The two pools of activity from Zn-chelate chromatography were separately further purified by Mono Q-HPLC. Zn-chelate pool 1 eluted as a single activity peak at 0.2 M NaCl on Mono Q-HPLC, corresponding to a single band of molecular weight ~100 kDa on SDS-PAGE as shown in Fig. 2. lane 2. In contrast, Zn-chelate pool 2 resolved into two peaks of activity at 0.2 M and 0.3 M NaCl on Mono Q-HPLC. Both peaks contained a major protein band of ~100 kDa and another band corresponding to ~180 kDa on SDS-PAGE as shown in Fig. 2 lane 3.

When material eluted in Zn-chelate pool 1 was analyzed by Superose 12-HPLC, the elution profile showed that the GPI-PLD eluted as a single peak with an apparent molecular weight of about 200 kDa as determined by molecular weight markers (Bio-Rad's Gel Filtration Standards), indicating that the enzyme exists as a dimer. However, when material in peak 2 of Zn-chelate pool 2 was analyzed by Superose 12-HPLC, three activity peaks were observed. Actual fractions were analyzed by SDS-PAGE and the results showed that the majority of GPI-PLD eluted in fractions corresponding to the region with molecular weights higher than 200 kDa suggesting that GPI-PLD eluted as higher molecular weight aggregates. The higher molecular weight aggregates (peak 1) exhibited higher specific activity toward VSG (~ 2.3×10^4 U/mg) than alkaline phosphatase (1.0×10^3 U/mg) as substrate.

Example 1 summarizes the purification of GPI-PLD from 2.5 liters of bovine serum utilizing the protocol described above, excluding the Superose 12-HPLC step. Dimer GPI-PLD purified from Zn-chelate, pool 1, showed the highest specific activity(6.3×10^5 and 4.5×10^5 U/mg against alkaline phosphatase and VSG, respectively). This represents a ~2,250 fold purification and an overall recovery of about 0.5% (Table 1).

The invention also relates to antibodies specific to GPI-PLD, fragments thereof or mutants thereof or mutants of GPI-PLD or fragments thereof substantially free from other proteins. These antibodies are suitable, e.g. for use in the purification of GPI-PLD active compounds as outlined below and in Example 1.

Production of Monoclonal Antibodies against GPI-PLD

Using a mixture of dimeric and aggregated GPI-PLD as immunogen, polyclonal antisera in mice against GPI-PLD were produced. All three immunized mice produced antibodies against the immunogen as determined by ELISA analysis. GPI-PLD activities were completely inactivated by antisera when purified or partially purified protein was used. When partially purified material was analyzed by immunoblotting, the 100-kDa protein was reactive with the antisera (data not shown).

To further confirm that the 100-kDa protein is GPI-PLD, monoclonal antibodies against the enzyme were

produced. Since serum contains GPI-PLD, hybridomas were grown in serum-free medium after fusion. We attempted to screen hybridomas by neutralization of GPI-PLD activity. Hybridoma supernatants were analyzed by ELISA. Clones secreting high levels of IgG antibodies were further screened in an immunodepletion assay against GPI-PLD activity. Twenty-four clones were obtained after subcloning. To further analyze the immunoprecipitated product, ^{125}I-labelled immunogen was used in an immunodepletion assay and the products analyzed by SDS-PAGE. The results show that the antibodies in the hybridoma supernatants selectively precipitated the GPI-PLD activity and the 100-kDa protein(data not shown).

The cells from ELISA-positive and immunodepletion-positive wells were subcloned. Twenty four clones were isolated and expanded as ascites tumors in BALB/cByJ mice. The monoclonal antibodies were purified and screened for their reactivity with the 100-kDa protein by immunoblotting analysis. Among them, nineteen showed strong reactivity. The immunoreactivity of the 100-kDa protein was dependent on antibody concentration and was saturated by excess antibody. Preabsorption of antibody with excess purified GPI-PLD diminished immunoreactivity.The purified antibodies were also screened for direct inhibition of GPI-PLD activity in solution. None of them inhibited GPI-PLD.

Purification of GPI-PLD by Immunoaffinity Chromatography

An experiment was carried out to determine which of the monoclonal antibodies would be most suitable for immunoaffinity chromatography. Antibodies with different affinities to GPI-PLD on ELISAs were separately coupled to CNBr-activated Sepharose. Crude bovine serum was loaded onto immunoaffinity columns and GPI-PLD activity was eluted by different reagents. The results showed that when weak affinity antibodies were used, bound GPI-PLD could be eluted with 3M $MgCl_2$ with about 60% recovery of activity. However, when high affinity antibodies were used, only a very small amount of GPI-PLD could be eluted with 3M $MgCl_2$. Although SDS-PAGE analysis indicated that most of the remaining bound protein could be eluted with 0.1 M glycine-HCl buffer (pH 2.8), enzymatic activity was lost. A weak affinity antibody was therefore chosen for immunoaffinity purification. The eluate from immunoaffinity chromatography gave a specific activity of about 9.75×10^3 U/mg, representing a 123 fold purification (see Table 2). Based upon this specific activity (assuming that the purified enzyme has a specific activity of 4.5×10^5 U/mg) and SDS-PAGE analysis a GPI-PLD purity of about 2% was calculated. See Fig. 3 lane 1.

Since the immunoaffinity-purified GPI-PLD could not be stably stored in 3 M $MgCl_2$, it was immediately diluted 6 fold with Buffer C (see Example 1) supplemented with 2.5 mM each $CaCl_2$ and zinc acetate. Calcium and zinc ions in the dilution buffer seemed to stabilize the enzyme activity, consistent with previous observations that the enzyme activity is dependent on calcium and zinc ions, but not Mg^{2+}. GPI-PLD in the diluted sample was then further purified on wheat germ lectin Sepharose. As shown in Table 2, a 10 fold purification was achieved with lectin Sepharose chromatography. On SDS-PAGE (Fig. 3, lane 2), the lectin Sepharose-eluate showed that although the major contaminating proteins were still in the sample, there was an enrichment of the 100-kDa GPI-PLD. GPI-PLD was further purified by Mono Q-FPLC. The elution profile showed that most of the activity eluted in a peak at 0.2 M NaCl, although a very small amount of activity also eluted at 0.3 M NaCl. When the samples were analyzed by SDS-PAGE, the major activity peak showed a single band with a molecular weight of 100 kDa. See Fig. 3 line 3. Samples in the minor activity peak also showed a very small amount (less than 10% of the total GPI-PLD recovered from the column) of 100-kDa protein together with other contaminating proteins. When the purified GPI-PLD was analyzed by Superose 12-HPLC, it eluted as a single peak with an apparent molecular weight of 200 kDa.

Table 2 in Example 1 summarizes the purification of GPI-PLD from 200 ml of bovine serum by the immunoaffinity chromatography protocol as described above. The specific activity of purified GPI-PLD from the immunoaffinity procedure was about the same as that obtained by the eight-step procedure. However, the overall recovery (26%) was much higher.

Characterization of GPI-PLD

The products of [^{3}H]myristate labelled VSG hydrolysis by the purified GPI-PLD were analyzed by thin layer chromatography on silica gel using two different solvent systems (chloroform:pyridine:70% formic acid, 50:30:7 or chloroform:methanol:glacial acetic acid:H_2O, 50:30:8:4). The ^{3}H-labelled product co-migrated with a dimyristyl phosphatidic acid standard. Other potential phospholipase products such as myristic acid and 1,2-dimyristoyl glycerol were not detectable (i.e. less than 5 % of recovered radioactivity). This result was obtained with both the dimeric form and the higher molecular weight aggregates.

The sensitivity of the enzyme activities to EGTA and 1,10-phenanthroline was studied. Table 3 shows that all enzyme activities are inhibited by EGTA and 1,10-phenanthroline, indicating that all forms of GPI-

PLD share a metal ion requirement. To further study the physical properties of dimer and aggregates of GPI-PLD, purified GPI-PLD was labeled with ^{125}I and the different forms of GPI-PLD were separated by Superose 12-HPLC. Each form of GPI-PLD was then rerun on Superose 12-HPLC. The results showed that the elution positions of these forms remained unchanged, indicating that the forms are not in equilibrium with each other.

The 100-kDa proteins in dimer and larger aggregated forms were isolated by preparative SDS-PAGE and subjected to amino-terminal sequence analyses. The results show that all forms of GPI-PLD share the same amino terminal sequence (H_2N-X-G-I-S-T-(H)-I-E-I-G-X-(R)- A-L-E-F-L--). A search within the GenBank and NBRF data bases using the computer programs TFASTA and SEARCH showed no strong sequence homology to that of any other known protein.

The primary structural relationships between these forms of GPI-PLD were also studied by comparing their tryptic peptide maps. Both samples were digested with trypsin, and cleavage products were separated by reverse phase HPLC on a C_8 column. The tryptic peptide maps are almost identical, indicating that the two forms of GPI-PLD represent either the same protein or are structurally very similar.

The tryptic peptides were further analyzed by protein microsequence analysis. Table 4 in Example 1 summarizes sequences derived from nine peak fractions.

The invention also relates to a method for producing a secretable protein from a eukaryotic cell said process comprises

a) transforming a host cell with a recombinant vector which codes for a polypeptide having GPI-PLD activity and with a recombinant vector coding for a GPI-anchored protein

b) culturing the transformed cell under appropriate conditions of growth so that both proteins are expressed and

c) isolating the protein from the culture medium the GPI-anchor of which is cleaved off.

Secretable proteins are produced by splicing the DNA sequence encoding the protein of interest together with a DNA sequence encoding for a peptide which signals the attachment of a glycosyl phosphatidylinositol-anchor (GPI-anchor) onto the protein. An example of such a C-terminal GPI signal peptide which signal for the attachment of a GPI-anchor onto a protein is a peptide with the C terminal 37 amino acids of CD16, namely:

Ser Thr Ile Ser Ser Phe Ser Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val

21 37

Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile.

The DNA sequence encoding the C-terminal GPI signal peptide is spliced onto the DNA sequence encoding the functional domain of the proteins forming the protein-GPI-anchor construct. The protein-GPI-anchor hybrid construct is then co-transfected into a eukaryotic cell such as a COS cell with a gene encoding a GPI-PLD such that both the protein-GPI anchor signal peptide hybrid construct and the GPI-PLD gene are expressed. A GPI anchor is attached to the Protein forming a GPI-anchor-protein; GPI-PLD enzymes cleave the anchor and the protein is secreted from the cell. Examples of proteins which could be produced and secreted in this way are CD4, ELAM-1, cytokine receptors such as p70 of the IL-2 receptor, members of the integrin and selectin families to name just a few.

The invention also relates to a process for cleaving proteins which are anchored to a cell by means of a glycosyl phosphatidylinositol anchor comprising administering to a cell culture in which the cell is growing GPI-PLD in combination with a suitable detergent such as CHAPS or NONIDET P40. To cleave off a GPI-PLD anchor, the proteins need not necessarily be anchord to a cell.

The present invention is further illustrated by the following examples.

Example 1

Purification and Characterization of PLD

Materials -- Bovine serum was from Pel-Freez Biologicals. PEG-5000 was from Polyscience Inc. Hydroxyapatite Ultrogel was from IBF Biotechnics. CNBr-activated Sepharose, Q Sepharose, wheat germ lectin-Sepharose and Sephacryl S-300 were from Pharmacia. IODO-BEADS, and immobilized (Fractogel

TSK HW-65F) iminodiacetic acid was from Pierce. CHAPS, (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), phenyl methyl sulfonyl floride (PMSF), Triton X-114 and 100, Nonidet P40 and goat anti-mouse IgG agarose were from Sigma. HRP conjugated goat $F(ab')_2$ anti-mouse IgG was from TAGO. Female Balb/c and Balb/cByJ mice were from Charles River Labs and Jackson Labs, respectively.

These and other sources of reagents described in the specification are provided merely for convenience and are not meant to be limiting on the invention.

Solutions -- Buffer A: 10 mM HEPES, pH 7.0, 0.15 M NaCl, 0.1 mM $MgCl_2$ and 0.01 mM zinc acetate; Buffer B: 50 mM Tris, pH 7.5, 0.1 M NaCl, 0.5 mM PMSF and 0.02% NaN_3; Buffer C: 50 mM Tris, pH 7.5, 0.1 M NaCl, 0.6% CHAPS and 0.02% NaN_3.

GPI-PLD assays -- For the eight-step purification of GPI-PLD, the enzyme activity was assayed as described in Low, M. G., and Prasad, A. R. S. (1988) Proc. Natl. Acad. Sci. USA, 85, 980-984 using GPI-anchored placental alkaline phosphatase as substrate. Typically, the alkaline phosphatase substrate (0.05 ml containing 1 vol of alkaline phosphatase, purified as described in Malik, A.-S. and Low, M. G. (1986) Biochem. J., 240, 519-527, 2 vol of 1% NP-40 and 2 vol of 0.2 M Tris-maleate, pH 7) was incubated with aliquots of samples in a total volume of 0.2 ml for 30 min at 37° C. The mixture was then diluted with 0.8 ml of ice-cold Buffer A. An aliquot (0.05 ml) was removed and mixed with 0.2 ml of Buffer A and 0.25 ml of 2% precondensed Triton X-114. After sampling a 0.1 ml aliquot for assay of total alkaline phosphatase activity, the mixture was incubated at 37° C for 10 min, centrifuged immediately at room temperature for 2 min and a 0.1 ml aliquot of the upper (aqueous) phase sampled. Alkaline phosphatase activity was determined. Anchor degradation was measured by comparing the activity in the upper phase (i.e., the degraded form) with that in the total mixture before phase separation. One unit is arbitrarily defined as the amount of enzyme hydrolyzing 1% of the alkaline phosphatase per min under the assay conditions described.

For the purification of GPI-PLD by immunoaffinity chromatography VSG biosynthetically labelled with [^{3}H]myristate was used as substrate. This was prepared by a modified procedure of a method described in Hereld, D., Krakow, J.L., Bangs, J. D., Hart, G. W., and Englund, P. T. (1986) J. Biol. Chem., 261, 13813-13819. Typically, T. brucei (MItat 117 or 118) were prepared from infected rats, labelled with -[^{3}H]myristic acid in vitro and the ^{3}H-labelled VSG was isolated. [^{3}H]Myristate-labelled VSG (4,000-5,000 cpm, 2 μg) was mixed with 0.02 ml of 0.2 M Tris maleate, pH 7.0, 0.02 ml of 1% NP-40 and 0.06 ml of H_2O. The substrate (0.1 ml) was then incubated with the GPI-PLD sample (0.1 ml) for 30 min at 37° C. The reaction was stopped by the addition of 0.5 ml of butanol that had been saturated with 1 M ammonium hydroxide. After vortexing, the phases were separated by centrifugation at 1,500 x g for 3 min. The upper(butanol) phase (0.35 ml) was withdrawn, mixed with 4 ml of scintillation fluid, and counted. One unit of GPI-PLD activity using VSG as a substrate is arbitrarily defined as the amount of enzyme hydrolyzing 1% of the [^{3}H]-myristate-labelled VSG per min.

To determine the substrate specificity of GPI-PLD, the products of [^{3}H]myristate-labelled VSG hydrolysis by purified GPI-PLD were analyzed by thin-layer chromatography as described by Low, M. G., and Prasad, A. R. S. (1988) Proc. Natl. Acad. Sci. USA, 85, 980-984. Hydrolysis of [^{3}H]choline-labelled phosphatidylcholine and [^{3}H]inositol-labelled PI was determined by substituting them for VSG in the incubation mixture described above. Water soluble radioactivity released from the phospholipids was determined as described by Low, M. G., Stiernberg J., Waneck, G. L., Flavell, R. A., and Kincade, P. W. (1988)J. Immunol. Methods, 113, 101-111.

Purification of GPI-PLD by the Eight-step Procedure

The purification steps are summarized in Table 1.

Table 1. Purification of GPI-PLD by the Eight-step Procedure

Step	Protein (mg)	PLD Activity[(a)] (U)	Sp. Activity (U/A_{280})	Purification Factor
Bovine Serum	146,855	4.1×10^7	2.8×10^2	1
PEG Sup.	67,365	3.1×10^7	4.6×10^2	1.6
Fast Q	3,686	7.5×10^6	2.0×10^3	7.1
S-300	920	3.1×10^6	3.4×10^3	12.1
Wheat Germ Lectin	106	2.2×10^6	2.1×10^4	75
Hydroxyapatite	14	1.6×10^6	1.1×10^5	392
Zn-chelate, pool 1	0.8	4.1×10^5	5.1×10^5	1,821
pool 2	2.5	3.0×10^5	1.2×10^5	(b)
Mono Q (Zn-chelate Pool 1)				
peak 1	0.1	6.3×10^4	6.3×10^5	2,250
Mono Q (Zn-chelate Pool 2)				
peak 1	0.28	4.0×10^4	1.5×10^5	(b)
peak 2	0.4	1.5×10^4	3.8×10^4	(b)

(a) GPI-PLD activity was determined using alkaline phosphatase as substrate.

(b) Since portions of GPI-PLD in the sample were present as aggregates with low specific activities, the degree of purification could not be determined accurately.

Bovine serum (2.5 l) was thawed at 4°C in the presence of 0.5 mM PMSF and 0.02% NaN_3. With stirring at 4°C, PEG-5000 was gradually added to a final concentration of 9%. The mixture was stirred for an additional hour and centrifuged at 10,000 x g for 25 min. The supernatant was collected and diluted with an equal volume of Buffer B. All subsequent purification steps were performed at 4°C except where noted.

The diluted supernatant was loaded at a flow rate of 30 ml/min onto a Q Sepharose column (9 x 10 cm) equilibrated in Buffer B. After washing with the equilibration buffer, GPI-PLD activity was eluted with a linear gradient of 0.1-1.0 M NaCl in 4 l of 50 mM Tris, pH 7.5, 0.02% NaN_3 and 0.5 mM PMSF. Fractions containing activity were pooled and concentrated by YM-10 (Amicon) membrane filtration to approximately 200 ml. The concentrate was loaded at a flow rate of 3.8 ml/min onto two (10 x 53 cm) S-300 columns in Buffer B, linked in tandem. The activity fractions were pooled, and NaCl and CHAPS were added to final concentrations of 0.2 M and 0.6%, respectively, to minimize protein aggregation. Half of the sample was loaded (flow rate: 17 ml/hr) onto a 40 ml (2.5 cm diameter) wheat germ lectin column equilibrated in 50 mM Tris, pH 7.5, 0.2 M NaCl, 0.02% NaN_3 and 0.6% CHAPS. After washing, the GPI-PLD activity was eluted with equilibrium buffer containing 0.3 M N-acetylglucosamine. The eluates from two runs were combined and concentrated to 10 ml. Nine volumes of 5 mM $NaPO_4$, pH 6.8, 0.4% CHAPS and 0.02% NaN_3 were added and the sample was loaded at room temperature (flow rate: 3 ml/min) onto a 4.2 x 22 cm column of hydroxyapatite Ultrogel in 5 mM $NaPO_4$, pH 6.8, 0.6% CHAPS and 0.02% NaN_3. GPI-PLD activity was collected in the wash fractions, and the contaminating proteins were eluted with 0.5 M $NaPO_4$, pH 6.8, 0.6% CHAPS, and 0.02% NaN_3.

GPI-PLD active fractions from hydroxyapatite agarose chromatography were pooled, concentrated by

YM-10 membrane filtration to 21 ml, and the pH adjusted with the addition of a 20-fold dilution of 1 M Tris HCl, pH 7.5. The sample was loaded onto a column (1.5 x 5.0 cm) of iminodiacetic acid on Fractogel TSK HW-65F chelated with zinc and equilibrated in Buffer C. The first peak of activity was collected in 10-15 bed volumes of wash with equilibration buffer and a sharper second peak of activity was eluted with 10 mM histidine in equilibration buffer.

The two Zn-chelate pools of activity were concentrated individually by YM-10 membrane filtration. Each sample (5 ml) was injected onto a Mono Q (HR5/5, Pharmacia) column equilibrated in Buffer C (without NaN_3) at room temperature. GPI-PLD activities were eluted at a flow rate of 1 ml/min with a gradient of 0.1-0.19 M NaCl in 50 mM Tris, pH 7.5, and 0.6% CHAPS in 6 min, followed by isocratic elution at 0.19 M NaCl for 5 min and a gradient of 0.19-0.4 M NaCl in 14 min. Under these conditions, the first Zn-chelate pool eluted as one activity peak at 0.2 M NaCl whereas the second Zn-chelate pool resolved into two peaks of activity at 0.2 M and 0.3 M NaCl.

GPI-PLD active fractions from Mono Q-HPLC were pooled, concentrated, and each sample (0.4ml) injected onto a Superose 12-HPLC (HR 10/30, Pharmacia) column equilibrated in Buffer C. Proteins were eluted at a flow rate of 0.3 ml/min and 0.5 ml fractions were collected.

Purification of GPI-PLD by Immunoaffinity Chromatography

Monoclonal antibody PLD 216.1 was coupled to CNBr-activated Sepharose at a final concentration of 1 mg/ml resin. Bovine serum (200 ml) was centrifuged at 16,000 x g for 20 min, and the supernatant diluted with 1.2 liters of Buffer B plus 0.5% NP-40. After filtering through a 0.22 µm membrane (Nalgene filter unit), the sample was loaded onto an immunoaffinity column (20 ml, 2.5 x 4 cm) at a flow rate of 30 ml/hr. The column was then washed with 400 ml of Buffer C and GPI-PLD eluted with 3M $MgCl_2$ in Buffer C. Active fractions were pooled (100 ml, 40 mg) and immediately diluted with 6 volumes of Buffer C plus 2.5 mM each $CaCl_2$ and zinc acetate. The sample was then loaded at a flow rate of 30 ml/hr onto a 20 ml (2.5 cm diameter) wheat germ lectin Sepharose column in 50 mM Tris, pH 7.5, 0.2 M NaCl, 0.6% CHAPS, 0.02% NaN_3 plus 2.5 mM each $CaCl_2$ and zinc acetate (equilibrium buffer). After the column was washed, the sample was eluted with 0.3 M N-acetylglucosamine in equilibrium buffer.

The pool (60 ml, 2.5 mg) of wheat germ lectin Sepharose-eluate was concentrated by YM-10 membrane filtration to about 15 ml and diluted with an equal volume of 50 mM Tris, pH 7.5, and 0.6% CHAPS. The sample was then loaded onto Mono Q-FPLC equilibrated in Buffer C (without NaN_3) at room temperature. GPI-PLD was eluted at a flow rate of 1 ml/min with a gradient of NaCl as described above.

The results of the single purification steps are summarized in Table 2.

Table 2. Purification of GPI-PLD by Immunoaffinity Chromatography

Step	Protein (mg)	PLD Activity[(a)] (U)	Sp. Activity (U/A_{280})	Purification Factor
Bovine Serum	8,333	6.6×10^5	7.9×10[(b)]	1
Immunoaffinity	40	3.9×10^5	9.8×10^3	123
Wheat Germ Lectin	2.5	2.4×10^5	1.0×10^5	1,266
Mono Q-FPLC	0.4	1.7×10^5	4.3×10^5	5,443[(c)]

(a) GPI-PLD activity was determined using [^{3}H]-VSG as a substrate.

(b) The specific activity of GPI-PLD in bovine serum shown in this Table is somewhat lower than that in Table 1 due to the variability in the commercially available material.

(c) The purification factor shown in this table is higher than that in Table 1 due to the lower specific activity of the starting material.

From Table 2, it can be estimated that GPI-PLD exists in bovine serum at a concentration of approximately 7 µg per ml.

Protein Determinations -- Protein concentration during purification was monitored by absorbance at 280 nm. In addition, the protein concentration of purified preparations was determined by the method of Bradford, M. (1976) Anal. Biochem., 72, 248-254 using Bio-Rad's protein assay reagent. One mg/ml of purified GPI-PLD corresponded to one optical density unit at 280 nm.

Tryptic Peptide Mapping -- The 100 kDa proteins in peaks 1 and 2 from Mono Q-HPLC were isolated by preparative SDS-PAGE. Proteins were recovered by electroelution in 67 mM N-ethylmorpholine acetate, pH 8.6, and 0.05% SDS as described in Hunkapillar, M. W., Lujan, E., Ostrader, F., and Hood, L. E. (1983) Methods Enzymol., 91, 227-236. After electroelution, proteins (100 ug) were reduced with 10 mM dithiothreitol for 2 h at 37° C and alkylated with 20 mM iodoacetic acid for 30 min at room temperature in the dark. Additional 10 mM dithiothreitol was added to the mixture to stop the reaction. Samples were lyophilized and proteins precipitated with acetone:acetic acid: triethylamine:water (85:5:5:5, by vol.). The precipitated proteins were washed twice with ice-cold acetone, dried and resuspended in 0.3 ml 0.1 M NH_4HCO_3, pH 8.0, and 0.5 mM $CaCl_2$. Samples were digested for 16 hr at 37° C with TPCK-treated trypsin (Cooper Biomedical, 6 µg total). The trypsin was added in three equal aliquots: the first at time zero, the second after 4 h, and the third after a 12 h incubation. Samples were acidified with formic acid to 15% and subjected to reverse phase HPLC on a C_8 column (Phase Separation Inc., 0.2 x 15 cm). Peptides were eluted (flow rate: 0.2 ml/min) with a gradient of acetonitrile (0-70%) in 0.1% trifluoroacetic acid.

Protein Sequencing-- Sequence analysis was performed using an Applied Biosystems (ABI, Foster City, CA) gas sequencer model 470A. PTH amino acids were identified "on line" with an ABI model 120A PTH analyzer using a reverse-phase C-18 column (2.1 x 220mm, ABI).

The sequences of the different tryptic peptides obtained this way are shown in Table 3.

Table 3. Sequences of Tryptic Peptides generated from GPI-PLD

Fragment (pmol)		Sequence
T_{56}	(~50)	S P F L V E Q F Q E Y F L G G L E D M A F X S T N I
T_{50}	(~15)	S I X E M F I G S X Q P L T H V
T_{44}	(~75)	V Y G Y F P X I C(Q)S I F T
	(~20)	M V A D V N X H X(L)G P E
T_{38}	(~80)	L G X A M T S A D L N Q D G Y G D L V V G A P G(Y)X(H)P G
T_{37}	(~150)	F G S A V A V L D F N V D G V P D L A V G A P S V G S(E)(K)
T_{35}	(~120)	A L E F L H L Q D G S I N Y K
	(~20)	H Q D A Y Q A G S V F P D S F
T_{34}	(~100)	H Q D A Y Q A G S V F P D S F Y P S I C E R
	(~50)	V S F L T M T L H Q G G S T R
T_{20}	(~325)	A Q Y V L I S P E A G S R
	(~205)	F G S S V(I)T V R
T_{18}a	(~110)	S N V T S (C P E E K) [F W Y L P] R

Tryptic fragments of the SDS-PAGE-purified 100-kDa protein were subjected to amino-terminal microsequence analysis on a gas phase sequencer. "X" indicates positions in the sequence where PTH-amino acids were not identified. The numbers in parentheses indicate the estimated amount of peptide sequenced. The amino acids in parentheses indicate the most likely assignments.

a The two peptides in this fraction exist in equimolar amounts and the first six residues in the assigned sequences may be exchanged with each other at the corresponding positions.

Preparation of Monoclonal Antibodies against GPI-PLD -- A female BALB/c mouse was immunized intraperitoneally with a mixture of two forms of mono Q-HPLC-purified GPI-PLD (60 μg protein) mixed 1:1 with Freund's complete adjuvant. Four weeks later, the mouse was boosted intraperitoneally with the same amount of immunogen in Freund's incomplete adjuvant. A test bleed was taken a week later and antiserum was checked by ELISA and by direct assay for neutralization of GPI-PLD activities.

Three days before fusion, the mouse was further boosted with 60 ug of immunogen by intravenous injection into the tail vein. Spleen cells from the mouse were fused with the myeloma cell line PAI-0 using procedures described by Thomas, P. E., Reik, L. M., Ryan, D. E., and Levin, W. (1984) J. Biol. Chem., 259, 3890-3899. Ten days after fusion, the cells were weaned into serum-free media (HL-1, Ventrex Laboratories) and 40 h later, supernatants were analyzed by ELISA for IgG production against the immunogen. ELISA positive cultures were expanded in serum-free media. Hybridoma exhibiting poor growth in serum-free media were grown in 0.5% horse serum. Under such conditions, endogenous horse serum GPI-PLD did not interfere with either the ELISA or immunodepletion assay. The established hybridoma cells were then grown as ascites tumors in pristane-primed BALB/cByJ mice. Anti-GPI-PLD monoclonal antibodies were purified from ascites fluids by caprylic acid and ammonium sulfate precipitation as described by Reik, L. M., Maines, S. L., Ryan, D. E., Levin, W., Bandiera, S., and Thomas, P. E. (1987) J. Immunol. Methods, 100,

123-130.

ELISA -- Non-competitive ELISA assays were run against mouse antiserum and culture supernatants as described by Thomas, P. E., Reik, L. M., Ryan, D. E., and Levin, W. (1984) J. Biol. Chem., 259, 3890-3899. Either immunogen or partially purified (wheat germ lectin- or Zn-chelate matrix-) GPI-PLD was coated onto 96-well polystyrene microtest plates. Binding of antibodies to GPI-PLD-coated plates was detected using HRP-conjugated second antibody and an appropriate chromogen as described by Thomas, P. E., Reik, L. M., Ryan, D. E., and Levin, W. (1984) J. Biol. Chem., 259, 3890-3899.

Immunodepletion assay -- Hybridoma supernatants were screened for their abilities to immunoprecipitate GPI-PLD activity. Culture supernatants (0.5 ml) were incubated with 50 µl of a 50% suspension of goat anti-mouse IgG-agarose for 1 h at 37 °C. BSA (0.5 mg) was added as a carrier protein. The beads were washed twice with 1 ml Buffer A plus 0.5% NP-40 and incubated with 40 µl wheat germ lectin-purified GPI-PLD diluted with Buffer A plus 1 mg/ml BSA. After 1 h at 37 °C, the beads were removed by centrifugation at 1,500 x g for 0.5 min, and the supernatants were analyzed for GPI-PLD activity using either alkaline phosphatase or ^{3}H-VSG as substrate.

Immunoblotting -- Immunoblotting was carried out as previously described by Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad Sci. USA, 76, 4350-4354. Antibodies (mouse antiserum or purified monoclonal antibodies) and second antibodies (HRP-goat F(ab')$_2$ anti-mouse IgG) were diluted in phosphate-buffered saline, 1% BSA, 5% normal goat serum, and 0.05% Tween 20. After several washes, peroxidase activity was detected with 4-chloro-1-napthol and hydrogen peroxide, as previously described by Nielsen, P.J., Manchester, K. L., Towbin, H., Gordon, J., and Thomas, G. (1982) J. Biol. Chem., 257, 12316-12321.

Immunoprecipitation -- Dimer GPI-PLD was iodinated with ^{125}I using IODO-BEADS. Free ^{125}I was removed with a desalting column (Econo-Pac 10DG, Bio-Rad). Hybridoma supernatants (0.25 ml each) were incubated with goat anti-mouse IgG-agarose beads (0.05 ml of 50% slurry) at 37 °C for 1.5 hr. BSA (0.2 mg) was added to each sample as a carrier protein during incubation. The mixtures were then centrifuged at 1,500 x g for 0.5 min, and the beads were incubated overnight at 4 °C with ^{125}I-labelled GPI-PLD (3.5 x 10^5 cpm) in 0.25 ml of 50 mM Tris, pH 7.5, 0.1 M NaCl, 0.5% NP-40, and 1 mg/ml BSA. The beads were removed by centrifugation and washed three times (0.8 ml each) with 50 mM Tris, pH 7.5, 0.1 M NaCl, and 0.5% NP-40. SDS-PAGE reducing sample buffer (40 µl) was added to the beads and aliquots (20 µl) were analyzed by SDS-PAGE. After electrophoresis, the gels were dried under vacuum and autoradiographed.

In summary, GPI-specific phospholipase D was purified from bovine serum by two different methods. The enzyme was initially purified by an eight-step procedure. Using the purified enzyme as immunogen, a panel of monoclonal antibodies against GPI-PLD were generated. Purified GPI-PLD from bovine serum was also accomplished by a simple procedure involving immunoaffinity chromatography, wheat germ lectin Sepharose and Mono Q-FPLC. The enzyme purified by the latter procedure is present as a dimer as analyzed by gel filtration-HPLC. However, the material purified by the eight-step procedure contains a mixture of dimer and higher molecular weight aggregates. These forms of GPI-PLD can be separated by Mono Q- and gel filtration-HPLC. On SDS-PAGE, the purified enzyme shows a single protein band with a molecular weight of 100 kDa. On native isoelectric focusing gels (data not shown), each form of GPI-PLD exhibits a common pI of about 5.6. Using VSG or alkaline phosphatase as substrate, the dimer exhibits a much higher specific activity than the higher aggregates.

When the 100-kDa protein and its tryptic peptides were subjected to amino acid sequencing analyses, the sequence data revealed no strong homologies to those of other known proteins except for the homology of two tryptic peptide sequences to each other and to the Ca^{2+} binding domains of calcium binding proteins. The discovery of two potential metal binding sequences is interesting in view of the data reported here that the enzyme activity is sensitive to the addition of divalent metal ion chelators, such as EGTA and 1,10-phenanthroline as shown in Table 4.

Table 4. Inhibitor Sensitivity of Two Forms of GPI-PLD

Enzyme	Inhibitor	Activity remaining (%)
GPI-PLD (dimer)		
	None	100
	EGTA (1.5 mM)	83
	EGTA (5.0 mM)	18
	1,10-phenanthroline (0.075 mM)	26
	1,10-phenanthroline (0.5 mM)	3
GPI-PLD (aggregates)		
	None	100
	EGTA (1.5 mM)	38
	EGTA (5.0 mM)	11
	1,10-phenanthroline (0.075 mM)	44
	1,10-phenanthroline (0.5 mM)	1.3

Mono Q-HPLC peaks 1 (A_{280}: 0.162) and 2 (A_{280}: 0.319) were diluted 400-fold with 10 mM HEPES, pH 7.0, and 0.15 M NaCl. An aliquot (0.1 ml) was incubated with various amounts of inhibitors for 1 hr at 4°C in a total volume of 0.11 ml. GPI-PLD activity was determined using VSG as substrate. Inhibitor concentrations refer to those present in the final incubation. Activities are expressed relative to those of controls.

Example 2

Cloning and Expression of Bovine Glycosyl Phosphatidyl Inositol-Specific Phospholipase D

Bovine liver cDNA libraries were screened with synthetic oligonucleotides corresponding to peptide sequences derived from purified bovine glycosyl phosphatidyl inositol-specific phospholipase D (GPI-PLD). Two overlapping clones were isolated that together predict the exact amino acid sequence of all eight tryptic fragments that had been sequenced. The DNA sequence of the two clones predicted a mature protein of 816 amino acids and an additional signal peptide of 23 amino acids. The deduced sequence contained eight potential N-linked glycosylation sites and at least four regions with sequence similarity to metal ion binding domains of members of the integrin family [Hynes, R.O. (1987) Cell, 48, 549-554]. These observations were consistant with the characterized GPI-PLD being 100 kd in size, glycosylated, and metal ion-dependent. The identification of the cloned cDNA was confirmed by two assays for biological activity. First, culture media and cell lysates of COS cells transfected with the gene showed phospholipase activity using ^{3}H-labelled GPI-anchored variant surface glycoprotein (VSG) of the African trypanosome as substrate in an in vitro assay. Analysis of the products from the in vitro VSG assays by thin layer chromatography showed that phosphatidic acid was a reaction product confirming that the phospholipase activity was that of phospholipase D. Second, COS cells transfected with a gene encoding GPI-anchored placental alkaline phosphatase (PLAP) released significant amounts of PLAP into the media when co-transfected with the GPI-PLD clone but not when transfected alone. These results suggest that GPI-PLD may play a role in the regulation of cell surface expression of GPI-anchored proteins in vivo.

The amino acid sequence of eight tryptic fragments from bovine GPI-PLD was used to design a set of

four degenerate oligonucleotide probes for the purpose of screening by DNA hybridization bovine DNA libraries. Because PLD activity had been detected in liver extracts, a liver cDNA library was initially screened. No positive clones were detected among the 5 x 10^5 clones screened. However, the screening of a bovine genomic library yielded one positive clone that hybridized to one of the four oligonucleotide probes. Partial DNA sequence analysis of this clone revealed an open reading frame that predicted exactly the sequence of the 22 amino acid tryptic fragment, T34. However, this coding sequence was in an exon that appeared to be only 79 bp in length. Instead of characterizing this genomic clone further, a second attempt at isolating a cDNA clone was made using two non-degenerate 30-mer oligonucleotides corresponding to the 79 bp exon sequence. In addition, a new bovine live cDNA library was constructed using random hexanucleotides to prime first strand synthesis. From 5 x 10^5 clones screened, two positive ones were isolated with the longer insert being 1.6 kb in length (clone pBJ1549). The complete sequence of the 1.6 kb insert was determined and shown to predict exactly the amino acid sequence of five of the eight tryptic fragments reported including (as expected) fragment T34 encoded by the genomic clone. Comparison of the deduced protein sequence to the N-terminal sequence of intact GPI-PLD revealed that the clone encoded the mature N-terminus of the protein (Cys^1 in Fig. 5). That means, that the initial translation product contains a 23 amino acid peptide.

Clone pBJ1549 was considered incomplete because 1) it encoded a protein of only 50 kd while a core protein of 80-100 kd was expected, 2) three of the eight tryptic sequences were not accounted for, and 3) an in-frame translation stop condon was not present. To isolate clones encoding the C-terminus, a liver cDNA library was screened with a nick-translated 400 bp fragment from the 3' end of pBJ1549. One clone was isolated that had a 1.1 kb insert (clone pBJ1644). Sequence analysis showed that the insert began at nucleotide 1450 of pBJ1549 and extended 1090 nucleotides in the direction of the C-terminus. The two clones had identical sequences in the 140 base region of overlap. The open reading frame identified in pBJ1549 continued in pBJ1644 until a stop codon at nucleotide 2557. The pBJ1644 insert encoded exactly the three tryptic fragments not encoded by pBJ1549. Together they encoded a 23 amino acid signal peptide and a 816 amino acid mature protein (90.2 kd) with eight possible N-linked glycosylation sites. These data indicated that these two clones combined contained the complete coding sequence for this protein.

Analysis of the deduced amino acid sequence revealed four regions of internal similarity (amino acids 379-402, 448-471, 511-534, and 716-739) that ranged from 21% to 54% identical (54% to 75% similar) to each other. A computer search in amino acid and nucleotide sequence databases revealed significant similarity of these repeats with the metal ion binding domains of the integrin alpha subunits. They share an aspartate-rich core sequence flanked by short conserved segments which are unique to the integrins. Apart from the absence of a glutamate residue, the core sequence DX(D/N)XDGXXD matches the EF-hand consenus motif characteristic of a number of Ca^{2+} and Mg^{2+} binding proteins such as calmodulin, troponin C, and parvalbuim. The observation that the gene reported here contains domains similar to metal ion binding domains of the integrins is consistent with the calcium requirements of GPI-PLD in enzymatic assays.

To express the cloned cDNAs and confirm that the encoded protein was GPI-PLD, the two inserts were first spliced together at the AccI site in their region of overlap and the resulting 2.6 kb cDNA ligated into the eukaryotic expression plasmid, pBC12BI (Cullen (1987) Methods in Enzymology 152, 684-704). The resulting plasmid, pBJ1682, was introduced into COS cells and expression confirmed by using a pool of monoclonal antibodies against the purified serum enzyme to perform 1) immunofluoresence of permeabilized cells (data not shown), and 2) Western blot analysis. While mock-transfected cell medium and lysate showed no detectable immunoreactive proteins by Western blot (lanes 1 and 2 in Figure 6), pBJ1682-transfected cells produced an immunoreactive protein detectable in both the medium and the lysate of sizes consistent with that of a glycosylated 90 kd core protein (lanes 3 and 4). However, the protein detected in the lysate migrated slightly faster than the protein secreted into the medium which in turn migrated faster then purified serum GPI-PLD. To demonstrate that these differences in migration were not due to differences in the types of sample (e.g. lysate vs. medium), pBJ1682-transfected cell medium or lysates were mixed with an equal volume of mock-transfected cell lysate or medium, respectively, prior to loading on the gel. The nature of these differences in size (estimated to be as much as 10 kd between lysate and purified serum proteins) may provide an important clue as to how the active form of this enzyme differs from its inactive form.

Culture media and cell lysates of the pBJ1682-transfected or mock-transfected COS cells were then prepared and incubated with ^3H-labelled GPI-anchored variant surface glycoprotein (VSG) to test for phospholipase activity. As shown in Fig, 7A, significant amount of phospholipase activity was detected in the medium of DNA-transfected cells while only background levels of activity was detected in mock-

transfected cells. After 46 hours in serum-free medium, the amount of phospholipase activity secreted reached 65 U/ml (approximately 0.15 µg/ml assuming that the COS cell secreted enzyme had the same specific activity as the purified bovine serum enzyme). Fig. 7B shows that from pBJ1682-transfected cells only a small amount of phospholipase activity was observed in the lysates compared to the media. These results indicated that the cloned gene did encode phospholipase enzyme and that most of the enzymatic activity was secreted from the cells.

Analysis of the reaction products of ^{3}H-labelled VSG hydrolysis assays confirmed that the phospholipase activity in DNA-transfected cells was that of phospholipase D (see Figure 8). The major ^{3}H-labelled product resulting from hydrolysis by purified serum GPI-PLD or the conditioned media from transfected cells co-migrated with dimyristoyl phosphatidic acid during thin-layer chromatography.

Transfected COS cell lysated and conditioned media were also examined for their specificities against non-GPI linked dipalmitoyl phosphatidylcholine substrate in the presence of ethanol. Neither PA nor phosphatidylethanol (a transphosphatidylation product of Phosphatidylcholine-specific PLD in the presence of ethanol) were detected by thin layer chromatography (data not shown) confirming that the cloned gene was the GPI-specific form of PLD.

Example 3

Transfection of COS Cell with a Gene encoding GPI-Anchored Protein alone and with a Gene encoding for GPI-PLD

To test for in vivo phospholipase activity against a GPI-anchored substrate, COS cells was transfected with a gene encoding GPI-anchored placental alkaline phosphatase (PLAP) alone or co-transfected with pBJ1682. Cell media and lysates were assayed for alkaline phosphatase activity. When COS cells were transfected with the PLAP cDNA alone, the majority of PLAP activity was detected in the cell lysate. This was consistent with PLAP being a GPI-anchored protein. When COS cells were co-transfected with both PLAP and pPJ1682, the amount of PLAP secreted into the medium was much higher then that of cells transfected with PLAP cDNA alone. The PLAP activity in the lysate of co-transfected cells was slightly higher than that of cells transfected with PLAP only, suggesting that in cotransfected cells GPI-anchored PLAP was constantly being synthesized and released by phospholipase activity. This was also supported by the fact that the total PLAP activity detected in the medium and lysates of co-transfected cells was consistently much higher than that in cells transfected with PLAP alone. In COS cells transfected with pBJ1682 DNA alone, only background levels of endogenous PLAP were detected in the medium or lysates. These results demonstrated that the cloned phospholipase could greatly affect the cell-surface expression of a GPI-anchored protein.

To test whether the GPI-PLD secreted from COS cells would hydrolyze cell-surface GPI-anchored PLAP, media from pBJ1682-transfected cells was incubated with PLAP-transfected cells and aliquots of media were assayed for PLAP activity after 1, 3, 8 and 24 hours of incubation. No PLAP activity was detected even though the media was active in the VSG assay both before and after the 24 hour incubation period.

As an alternative means of determining whether GPI-anchored proteins were hydrolysed by GPI-PLD, the cell culture supernatants of cotransfected COS cells were examined by immunoprecipitation following labelling with 3H-ethanolamine. If GPI-anchored proteins were actually being hydrolyzed by GPI-PLD, then the hydrolyzed products would be expected to maintain the 3H-ethanolamine moietoydrolytic products derived by proteolysis, which would not contain this group. Both PLAP and CD16 can be released from GPI-PLD co-transfected cells in a form which still contains an ethanolamine residue. These results eliminate the possibility that the released proteins are proteolytically derived products, and demonstrate that at least two different GPI-anchored proteins can be released by GPI-PLD.

Example 4

Molecular Cloning of the Human Liver Phospholipase D Gene

Tryptic peptide fragment sequences of a bovine GPI-PLD and DNA sequence from a partial bovine genomic clone were available. Using this information, a pair of oligonucleotides (#1s, #1a) were designed to search for a human source of PLD mRNA by the polymerase chain reaction. In liver, the presence of the message was detected by a 81-bp amplicon. Based on bovine cDNA sequences, primers were prepared (#5s, #4a) to amplify the 1.2kb fragment corresponding to the 5' half of the phospholipase D transcript from

human liver first-strand cDNA.

A partial human GPI-PLD cDNA clone was isolated by library screening. Human liver polyA$^+$ mRNA was primed using oligo-dT and size selected. EcoRI-linkered cDNA was cloned into the lambda-ZAP II vector (Stratagene). This library of 2.5 million recombinants was screened unamplified in duplicate with the bovine cDNA (at low stringency) and the 1.2-kb human GPI-PLD amplicon (at high stringency). A positive clone was identified by both probes and the insert was sequenced. This partial cDNA clone (nucleotide 688-1247) encoded 186 amino acid residues (230-416).

Since the mature amino terminus of human GPI-PLD was found to be highly conserved with that of the bovine protein (11 amino acids identical of the first 12) and partial C-terminus sequence of a human pancreas PLD cDNA was available, two oligonucleotides (#5s, #9a) were made to amplify a 2.5-kb amplicon from human liver first-strand cDNA. The segment corresponds to sequence coding for the mature human phospholipase D gene product. The amplicon was cloned into the vector pRcCmV (Invitrogen) and pBC12BI-derived vectors for expression in mammalian cells.

The DNA sequence coding for the mature human GPI-PLD protein was obtained from two independently isolated clones of the 2.5 kb amplicon, the partial cDNA clone and the 1.2 kb amplicon (described above; see Fig. 9). The predicted peptide sequence is 817 amino acid and 82% identical to the bovine sequence.

To clone the signal peptide of human GPI-PLD, an oligonucleotide (#5RT) was designed to prime cDNA synthesis from liver polyA + RNA. An adaptor-linker was ligated to the ends of the cDNA which was then subjected to two rounds of PCR using an adaptor primer and the oligonucleotides #5amp followed by #237. A 300 bp amplicon detected by #5s on a Southern blot was subcloned and the sequence of seven clones were determined. The signal peptide of human liver GPI-PLD is 24 amino acids long and the sequence matched closely to that of the bovine GPI-PLD (14 amino acids identical). The human liver signal peptide was joined to the mature protein coding region via a HpaI site in the pRcCMV expression construct.

The following oligonucleotides and PCR-conditions were used in the procedure described above.

Oligonucleotides

# Sequence	nucleotide positions
1s: CTGTTACTTAGGCACCAGG	bovine 85-103
1a: CTCTCTCACAGATGCTAGG	bovine 144-162
5s: TGTGGCCTTTCGACACACATAGAAATAGG	human 1-29
4a: ACGCGCCCCACGTGAATGCGGCCTGGGTG	bovine 1150-1178
9a: TCAATCTGAGCCAAGGCTATAGAC	human 2430-2453
5RT: GAATCCTTGTTCAATG	human liver 411-426
5amp CTGCTACCATATGAGAAGTA	human liver 388-369
237: TATGCATCCTGGTCTTCT	human liver 182-165

Plasmid or genomic DNA, single stranded cDNA, or lambda phage have been used as template in PCRs. A 50µl-reaction contains 10mM Tris-HCl pH8.3 at 25°c, 1.5 mM $MgCl_2$, 50mM KCl, 0.01%(w/v) gelatin, template DNA, a pair of oligonucleotide primers (50pmol each), 2.5 units Taq DNA polymerase (Cetus-Perkin Elmer) and 200uM of dATP, dCTP, dGTP, dTTP. Template DNA was denatured at 94° C for 7 minutes. The amplification was carried out in a Perkin-Elmer thermocycler for 25-35 cycles. Each cycle consists of a denaturation step set at 94°c for 1 min, an annealing step at 55°c for 2 min and an extension step at 72°c for 3 min. The denaturation step of the first cycle was extended to 7 min and an extra 72°c, 10 min extension step was included at the end of the cycles. The PCR products were analyzed in an 1-4% agarose gel. Amplified DNA was excised from gel, purified on glass beads (Geneclean) and subcloned into the HincII site of the general purpose cloning vector pBS (Stratgene).

The Library Construction & Screening was performed as follows:

Human liver mRNA was purchased from Clontech Labs and cDNA was synthesized by the procedure of Gubler and Hoffmann (1983). In a 50µl reaction (50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$, 10mmM DTT, 0.5mM dATP, 0.5mM dCTP, 0.5mM dGTP, 0.5mM dTTP), lug of mRNA primed with 1.25µg oligo-dT was converted into single stranded cDNA using the RNAseH negative MMLV-reverse transcriptase (BRL). The reaction was incubated at 37°c for 1 hour. The yield was monitored by adding 10µCi ^{32}P-dCTP to the reaction and measuring incorporated radioactivity after TCA precipitation.

Second strand synthesis was carried out as follows using buffers from the Amersham cDNA synthesis and cloning kit. The 250µl-reaction contains 50µl of 1st strand synthesis reaction, 93.5 µl 2nd strand synthesis buffer, 4U RNaseH, 115U DNA polymerase I and 91.5µl water. The synthesis was carried out at 14°C for 1 hr, then at room temperature for 1 hr followed by an 10-min incubation at 70°C. 2 µl T4 DNA polymerase (4 U/µl) was added and the mix incubated for 10 min at 37°C. The yield of the reaction was estimated by counting TCA precipitated cDNA. Purified double-stranded cDNA was methylated in a 20-µl reaction containing 4µl of M buffer, 1x s-adenosylmethionine and 30U of EcoRI methylase. The mixture was incubated at 37°C for 1 hr and then 10 min at 70°C to inactivate the enzyme. EcoRI linkers (1.5µg) were ligated to methylated cDNA (1.5µg) in an overnight reaction at 15°C in 50mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and T4 DNA ligase. The linkered cDNA was digested with EcoRI (100U) in a 100µl reaction for 5 hrs at 37°C. Digested cDNA was then size fractionated in a Sephacryl S500 column and high molecular weight fractions were pooled and purified.

The gene library was constructed in the vector, lambda ZAPII (Strategen). cDNA was ligated to the EcoRI-digested phosphorylated vector overnight at 14°C in a 10µl reaction containing T4 DNA ligase and its buffer. Ligated cDNA was packaged into phage using the Gigapack kit under conditions suggested by Stratagene.

A library of 2.5 million clones was generated and plated out on XL-1 blue cells and duplicate set of filters were lifted. The procedure for plaque hybridization of Benton & Davis (1977) Science 196, 180-182, was followed. A radioactive ^{32}P labelled DNA probe ($2.5x10^8$cpm/µg) was prepared by the random priming method (Feinberg & Vogelstein (1984) Anal. Biochem. 137, 266-267). Hybridization was carried out in 6xSSC, 0.1% SDS, 5x Denhardt's, 100µg/ml salmon sperm DNA, 25-50% formamide at 42°c overnight. The filters were washed in 0.1-2x SSC, 0.1% SDS at 37°c (low stringency) or 55°c (high stringency) before autoradiography.

DNA Sequencing

Double stranded plasmid DNA was sequenced according to the procedure described in the Sequenase (USB) manual. Figure 9 shows the nucleotide sequence and translated amino acid sequence of the human liver GPI-PLD. Figure 10 shows the alignment of amino acid sequence of the human and bovine liver GPI-PLD mature protein.

Example 5

Isolation and Characterization of a Human Pancreatic Phospholipase D cDNA Clone

Total RNA was isolated from a human pancreas tumor as described by Gubler et al., (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4311-4314. Poly A^+ RNA was selected resulting in a yield of 2.5%(w/w) relative to the total amount of RNA input. A cDNA library was constructed in λgt11 and amplified according to procedures published in Sambrook,J., Fritsch,E.F. and Maniatis, T., Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Laboratory Press 1989. The cDNA library was screened using the bovine GPI-PLD nick-translated cDNA as a probe [see Kocha,J. et al. (1986) Cell 44, 689-696] under conditions of reduced stringency (25% formamide). Two positive clones were plaque purified, cDNA inserts were subcloned in pGem3z (Promega Biotec) and their sequence determined using the dideoxy sequencing technique as recommended by the manufacturer of sequenase (United States Biochemical Corp.) The sequence of clone pJJ1935a is shown in Fig. 11 and begins at nucleotide 1 (corresponding to nucleotide 1609 of the bovine GP-PLD nucleotide). The sequence of clone pJJ1939 begins at nucleotide position 410 of pJJ1935a and is identical to pJJ1935a.

Analysis of the partial amino acid sequence of the human pancreas GPI-PLD reveals a high level of identity (81%) when compared to the bovine amino acid sequence, and 84% identity at the nucleotide level.

Example 6

Novel Process for the Production of recombinant, secretable Proteins

Other proteins that are normally not GPI-anchored can be made to be GPI-anchored by modifying their gens to encode the signal sequence for GPI-attachment at their 3' ends. If cells are transfected with both this modified gene and the gen for GPI-PLD, the protein gets secreted.

To determine if the principle of GPI-anchor protein secretion could be applied in general to other proteins, the GPI-anchor of CD16 was tranfered to other proteins, and their expression was monitored in the presence or absence of GPI-PLD. A DNA fragment encoding the portion of CD16 that signals GPI attachment [See Scallon et al., Proc. Natl. Acad. Sci. USA 86,5079-5083 (1989), Selvaraj et al., Nature 333, 565-567 (1988) and Simmons et al., Nature 333,568-570 (1988)], namely the C-terminal 37 amino acids.

Ser Thr Ile Ser Ser Phe Ser Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val
21 37
Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile,

was spliced to DNA encoding the extracellular domains of hte Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1) [See Bevilacqua, M.P. et al., (1989) Science 243, 1160-1165]; and to DNA encoding the extracellular domains of the p70 subunit of the IL-2 receptor (Hatakeyama, M. et al., (1989) Science 244, 551-556) by general methods common to the art.

Specificallly for the ELAM-1, two independent constructs were made using PCR technology, namely, ELAM-1-1-GPI and ELAM-1-2-GPI. For ELAM-1-1-GPI the oligonucleotide 5'-TTTGATCATTCTCTCAGCTCTCACTTTG-3' (5' sense primer) and 5'-TGGTCGACTCAGTGGGAGCTTCACAGGT-3' (3' anti-sense primer) were used to generate an amplicon. This amplicon was then digested with the restriciton enzymes BelI and SalI and contained the ELAM extracellular coding sequences (amino acids 15-532) used for the ELAM-1-GPI construct. For ELAM-1-2-GPI, the oligonucleotides 5'-TTTGATCATTCTCTCAGCTCTCACTTTG-3' (5' sense primer) and 5'-TAGTCGACACAATTTGCTCACACTTGAG-3' (3' anti-sense primer) were used to generate an amplicon. This amplicon was then digested with the restriction enzymes BelI and SalI and contained the ELAM-1 extracellular coding sequences (amino acids 15-157) used for the ELAM-1-2-GPI construct.

For p70-GPI the oligonucleotides 5'-ACGTCGACGTGTCCTTCCCAAGGGCTGC-3 (3' anti-sense primer) and 5'-CCGGATCCTGTCCTGGCGTCTGCCCCTC3' (5' sense primer) were used to generate an amplicon. This amplicon was digested with the restriction enzymes BamHI and SalI and contained the p70 extracellular coding sequences (amino acids 21-214) used for the p70-GPI construct.

The C-terminal GPI signal peptide from CD16 was also isolated by using PCR technology. The oligonucleotides 5'-GTGTCGACCATCTCATCATTCTCTCCA-3' (5' sense primer) and 5'-AGTGTTTGTGTAGCTCTGAAACTT-3" (3' anti-sense primer) were used to generate an amplicon, which was digested with the restriction enzymes SalI and StuI (internal site in the amplicon) and encoded amino acids 180-2167 of the CD16 protein. To generate the various GPI chimeric constructs, the protein coding regions of the protein of interest were ligated to the CD16 GPI-anchor sequences and in turn ligated into the eukaryotic expression vector pBC12BI (which had been digested with BamHI and SmaI). The different GPI-constructs were identified by colony hybridization, and verified by restriction enzyme analysis and DNA sequencing.

To determine if the hybrid GPI-proteins were secreted when cotransfected with the GPI-PLD, COS cells were transfected with ELAM-1-1-GPI (Fig. 12A), ELAM-1-2-GPI (Fig. 12 B) or p70-GPI (not shown) in the presence or absence of pBJ1682. Two days after transfection, the cells were metabolicially labelled with ^{35}S-cysteine for two hours. The cell media or extracts were immunoprecipitated using antibodies directed against the protein of interest, fractionated by SDS-PAGE and visualized by fluorography. The protein of interest was detected in all of the cell extracts examined but was only found in the supernatant when the GPI-PLD construct was co-tranfected. These results demonstrate that a GPI-anchor can be attached to a protein which his not normally GPI-anchored, and that this novel hybrid protein is secreted if it is expressed in the presence of the GPI-PLD enzyme. Such a secreted protein may be therapeutically relevant in the traeatment of various disesase depending on the hybrid protein which is used.

The teachings of all of the references cited in the present application including those listed below are

incorporated herein by reference.

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Claims

1. The protein glycosyl phosphatidylinositol-specific phospholipase D (GPI-PLD) or biological activ fragments thereof, substantially free from other proteins.

2. A glycosyl phosphatidylinositol-specific phospholipase D active compound which is a mutant of a compound as claimed in claim 1.

3. A compound as claimed in claims 1 or 2, wherein the protein of claim 1 is bovine liver GPI-PLD.

4. A compound as claimed in claim 3, wherein the bovine liver GPI-PLD has the amino acid sequence as set forth in Figure 5.

5. A compound as claimed in claim 1 or 2, wherein the protein of claim 1 is human liver GPI-PLD.

6. A compound as claimed in claim 5, wherein the human liver GPI-PLD has the amino acid sequence as set forth in Figure 9.

7. A compound as claimed in claim 1 or 2, wherein the protein of claim 1 is human pancreatic GPI-PLD.

8. A compound as claimed in claim 7, wherein the human pancreatic GPI-PLD has the partial amino acid sequence as set forth in Figure 11.

9. A glycosyl phosphatidylinositol-specific phospholipase D active fragment of a compound as claimed in anyone of claims 3-8.

10. A glycosyl phosphatidylinositol-specific phospholipase D active mutant of a compound as claimed in anyone of claims 3-8.

11. A glycosyl phosphatidylinositol-specific phospholipase D active fragment of a mutant of a compound as claimed in anyone of claims 3-8.

12. A polynucleotide coding for a protein as claimed in anyone of claims 1-11 or the complementary strand thereto.

13. A recombinant vector containing a polynucleotide as claimed in claim 12 operatively linked to an expression DNA-sequence.

14. A recombinant vector of claim 13 which is a plasmid or viral vector capable of replication in a eukaryotic or prokaryotic cell.

15. A prokaryotic or eukaryotic host cell transformed or transfected with a vector as claimed in anyone of claims 13 or 14.

16. A process for producing a protein as claimed in anyone of claims 1-11, comprising culturing a host containing a recombinant vector as claimed in anyone of claims 13 or 14 under appropriate conditions of growth so that said protein is expressed and isolating said protein.

17. A process of claim 16, wherein the host are CHO cells.

18. A process for secreting a protein from a cell which process comprises
a) transforming a host cell with a recombinant vector as claimed in anyone of claim 13 or 14 and with a recombinant vector coding for a GPI-anchored protein
b) culturing the transformed cell under appropriate conditions of growth so that both proteins are expressed and
c) isolating the protein from the culture medium the GPI-anchor of which is cleaved off.

19. A process claim 18, wherein the GPI-anchored protein is formed by splicing a C-terminal GPI-signal peptide to a protein of interest

20. A process of claim 19, wherein the C-terminal GPI-signal peptide is derived from the CD16 protein.

21. The use of a protein as claimed in anyone of claims 1-11 for cleaving of proteins which are attached to a glycosyl phosphatidylinositol anchor, characterized in that a protein attached to a GPI-anchor is cleaved from the GPI-anchor by reaction with said protein and a suitable detergent.

22. Antibodies specific to a protein as claimed in anyone of claims 1-11.

FIG 1

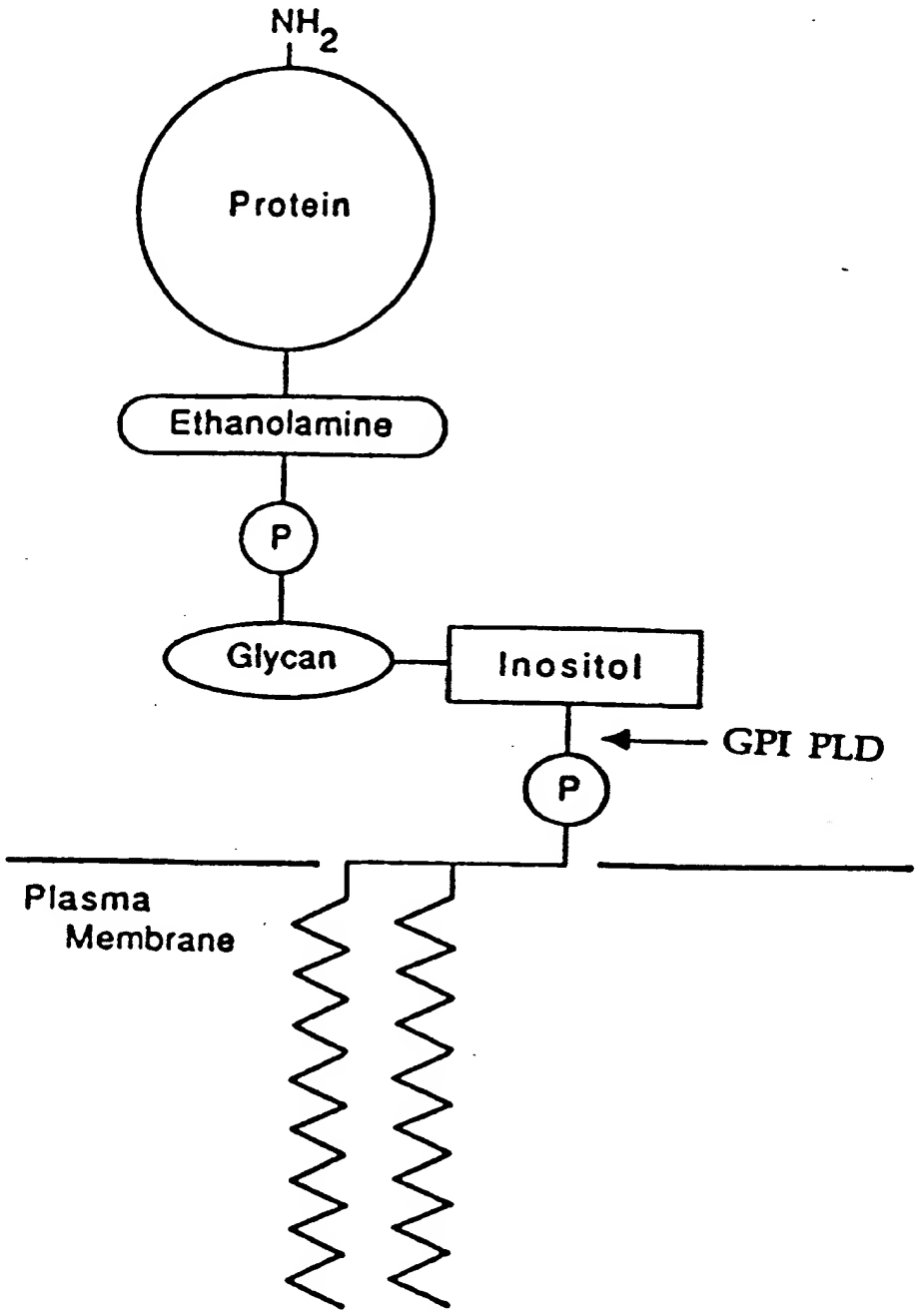
NH2
Protein
Ethanolamine
P
Glycan
Inositol
GPI PLD
P
Plasma
Membrane

FIG 2

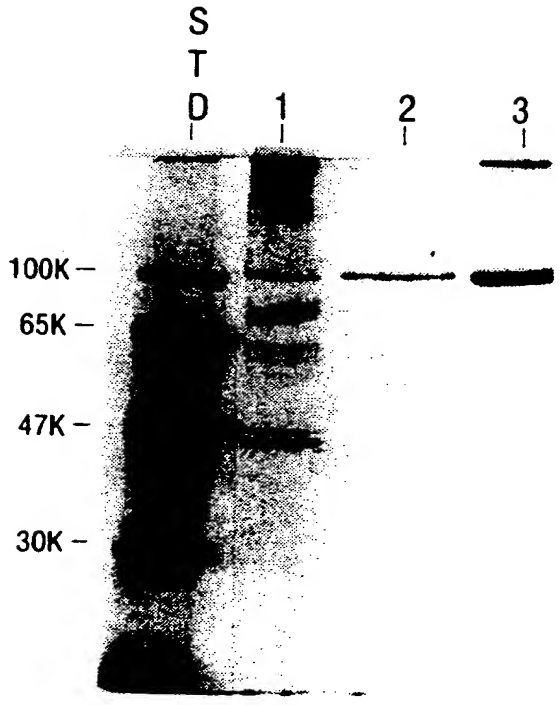

FIG 3

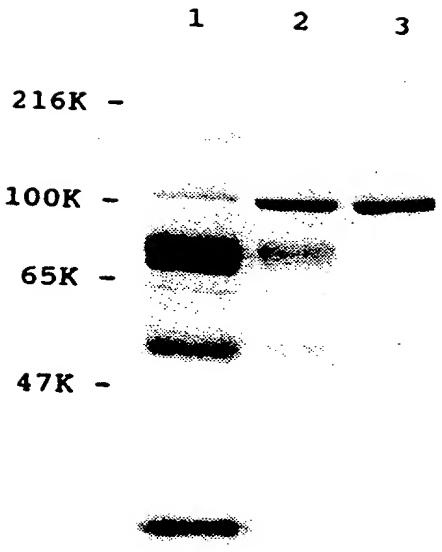

FIG 4

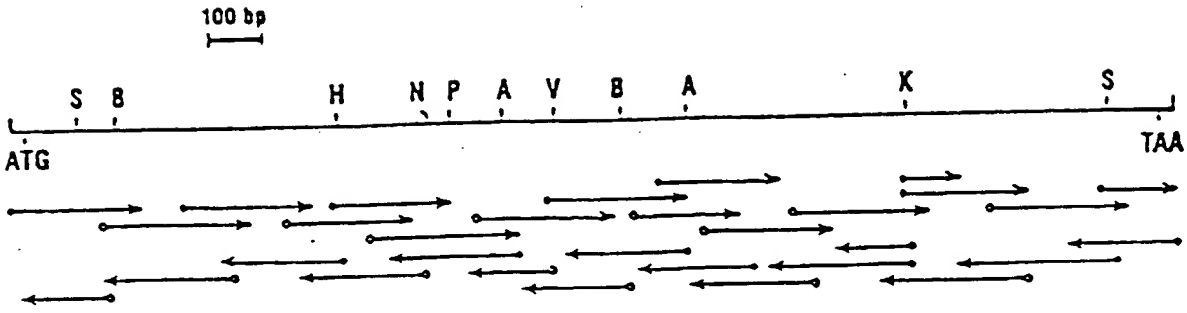

FIG 5

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                                                  Met Ser Ala Phe Arg Phe Trp Ser Gly Leu   -14
GCATTGCTCGTCACCATAGGAGCGGGGAGTAATGAGAGC ATG TCT GCT TTC AGA TTC TGG TCA GGA CTG    69

Leu Met Leu Leu Gly Phe Leu Cys Pro Arg Ser Ser Pro Cys Gly Ile Ser Thr His Ile    7
CTG ATG CTA CTG GGC TTC CTC TGC CCT AGA AGT TCA CCA TGT GGC ATT TCG ACA CAC ATA   129

Glu Ile Gly His Arg Ala Leu Glu Phe Leu His Leu Gln Asp Gly Ser Ile Asn Tyr Lys   27
GAA ATA GGA CAC AGA GCT CTG GAG TTT CTC CAC CTT CAG GAT GGG AGT ATT AAC TAC AAA   189

Glu Leu Leu Leu Arg His Gln Asp Ala Tyr Gln Ala Gly Ser Val Phe Pro Asp Ser Phe   47
GAG CTG TTA CTT AGG CAC CAG GAT GCA TAT CAG GCT GGA TCC GTG TTT CCT GAC TCA TTT   249

Tyr Pro Ser Ile Cys Glu Arg Gly Gln Phe His Asp Val Ser Glu Ser Thr His Trp Thr   67
TAC CCT AGC ATC TGT GAG AGA GGA CAA TTC CAT GAC GTG TCA GAG AGC ACT CAC TGG ACT   309

Pro Phe Leu Asn Ala Ser Val His Tyr Ile Arg Lys Asn Tyr Pro Leu Pro Trp Asp Glu   87
CCA TTT CTT AAC GCA AGT GTT CAT TAT ATC CGG AAG AAC TAT CCT CTT CCC TGG GAT GAG   369

Asp Thr Glu Lys Leu Val Ala Phe Leu Phe Gly Ile Thr Ser His Met Val Ala Asp Val   107
GAC ACA GAG AAA TTG GTA GCT TTC TTG TTT GGA ATT ACG TCT CAC ATG GTG GCT GAT GTC   429

Asn Trp His Ser Leu Gly Ile Glu Gln Gly Phe Leu Arg Thr Met Ala Ala Ile Asp Phe   127
AAC TGG CAT AGC CTG GGT ATT GAA CAA GGA TTC CTT AGG ACG ATG GCT GCC ATT GAT TTT   489

His Asn Ser Tyr Pro Glu Ala His Pro Ala Gly Asp Phe Gly Gly Asp Val Leu Ser Gln   147
CAC AAC TCC TAT CCC GAG GCA CAT CCG GCT GGT GAT TTC GGA GGA GAC GTG TTG AGC CAG   549

Phe Glu Phe Lys Phe Asn Tyr Leu Ser Arg His Trp Tyr Val Pro Ala Glu Asp Leu Leu   167
TTC GAG TTT AAA TTT AAT TAC CTC TCA CGG CAC TGG TAT GTG CCT GCT GAA GAT CTC CTG   609

Gly Ile Tyr Arg Glu Leu Tyr Gly Arg Ile Val Ile Thr Lys Lys Ala Ile Val Asp Cys   187
GGA ATT TAT AGA GAA CTC TAC GGC CGA ATA GTC ATC ACC AAA AAA GCC ATT GTT GAC TGT   669

Ser Tyr Leu Gln Phe Leu Glu Met Tyr Ala Glu Met Leu Ala Ile Ser Lys Leu Tyr Pro   207
TCA TAC CTT CAA TTC TTG GAA ATG TAT GCG GAG ATG TTA GCT ATT TCC AAG CTT TAT CCC   729

Thr Tyr Ser Val Lys Ser Pro Phe Leu Val Glu Gln Phe Gln Glu Tyr Phe Leu Gly Gly   227
ACT TAT TCT GTA AAA TCC CCA TTT TTG GTG GAA CAA TTT CAA GAA TAC TTC CTA GGA GGG   789

Leu Glu Asp Met Ala Phe Trp Ser Thr Asn Ile Tyr His Leu Thr Ser Tyr Met Leu Lys   247
CTG GAA GAT ATG GCG TTT TGG TCC ACT AAT ATT TAC CAT CTG ACA AGT TAC ATG TTA AAG   849

Asn Gly Thr Ser Asn Cys Asn Leu Pro Glu Asn Pro Leu Phe Ile Thr Cys Gly Gly Gln   267
AAC GGG ACC AGT AAC TGC AAC CTC CCT GAG AAC CCT CTG TTC ATC ACA TGT GGC GGT CAA   909

Gln Asn Asn Thr His Gly Ser Lys Val Gln Lys Asn Gly Phe His Lys Asn Val Thr Ala   287
CAA AAC AAC ACC CAT GGC TCA AAA GTA CAG AAA AAT GGT TTT CAT AAA AAT GTG ACT GCA   969

Ala Leu Thr Lys Asn Ile Gly Lys His Ile Asn Tyr Thr Lys Arg Gly Val Phe Phe Ser   307
GCC CTA ACT AAA AAT ATT GGA AAG CAT ATA AAC TAT ACC AAA AGA GGA GTG TTC TTT AGT   1029

Val Asp Ser Trp Thr Met Asp Phe Leu Ser Phe Met Tyr Lys Ser Leu Glu Arg Ser Ile   327
GTG GAT TCC TGG ACC ATG GAT TCC TTA TCC TTC ATG TAC AAG TCT TTG GAG AGG AGT ATA   1089

Arg Glu Met Phe Ile Gly Ser Ser Gln Pro Leu Thr His Val Ser Ser Pro Ala Ala Ser   347
CGG GAG ATG TTT ATT GGC AGC TCT CAG CCA CTG ACA CAT GTT TCT AGC CCC GCA GCA TCT   1149

Tyr Tyr Leu Ser Phe Pro Tyr Thr Arg Leu Gly Trp Ala Met Thr Ser Ala Asp Leu Asn   367
TAC TAC TTG TCA TTT CCC TAC ACA AGG CTT GGT TGG GCA ATG ACT TCA GCT GAT CTC AAC   1209

Gln Asp Gly Tyr Gly Asp Leu Val Val Gly Ala Pro Gly Tyr Ser His Pro Gly Arg Ile   387
CAG GAT GGA TAC GGT GAC CTG GTG GTG GGT GCC CCT GGC TAC AGC CAC CCA GGC CGC ATT   1269

His Val Gly Arg Val Tyr Leu Ile Tyr Gly Asn Asp Leu Gly Leu Pro Arg Ile Asp Leu   407
CAC GTG GGG CGC GTG TAC CTC ATC TAT GGC AAT GAC CTG GGC TTG CCC CGT ATC GAC CTG   1329

Asp Leu Asp Lys Glu Ala His Gly Ile Leu Glu Gly Phe Gln Pro Ser Gly Arg Phe Gly   427
GAC CTG GAC AAG GAG GCC CAC GGG ATC CTG GAG GGT TTC CAG CCC TCA GGT CGA TTT GGC   1389
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FIG 5 (continued)

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Ser Ala Val Ala Val Leu Asp Phe Asn Val Asp Gly Val Pro Asp Leu Ala Val Gly Ala   447
TCG GCT GTG GCT GTG CTA GAC TTT AAC GTG GAT GGC GTG CCT GAC CTG GCG GTG GGA GCC  1449

Pro Ser Val Gly Ser Glu Lys Leu Thr Tyr Thr Gly Ala Val Tyr Val Tyr Phe Gly Ser   467
CCC TCG GTG GGC TCC GAG AAG CTC ACA TAC ACA GGT GCA GTG TAT GTC TAC TTC GGT TCC  1509

Lys Gln Gly Gln Leu Ser Ser Ser Pro Asn Val Thr Ile Ser Cys Gln Asp Thr Tyr Cys   487
AAA CAA GGA CAA CTA TCT TCT TCC CCC AAC GTC ACC ATC TCT TGC CAG GAT ACC TAC TGT  1569

Asn Leu Gly Trp Thr Leu Leu Ala Ala Asp Val Asn Gly Asp Ser Glu Pro Asp Leu Val   507
AAC TTG GGC TGG ACC CTC CTG GCG GCA GAT GTG AAT GGA GAT AGT GAA CCG GAC CTG GTG  1629

Ile Gly Ser Pro Phe Ala Phe Gly Gly Gly Lys Gln Lys Gly Ile Val Ala Ala Phe Tyr   527
ATT GGC TCC CCT TTT GCT CCA GGT GGA GGG AAA CAG AAG GGA ATT GTG GCT GCA TTT TAC  1689

Ser Gly Ser Ser Tyr Ser Ser Arg Glu Lys Leu Asn Val Glu Ala Ala Asn Trp Met Val   547
TCT GGC TCC AGT TAC AGC AGC CGA GAA AAG CTG AAT GTG GAG GCT GCC AAC TGG ATG GTG  1749

Lys Gly Glu Glu Asp Phe Ala Trp Leu Gly Tyr Ser Leu His Gly Val Asn Val Asn Asn   567
AAA GGC GAG GAG GAC TTT GCT TGG TTG GGG TAC TCC CTT CAC GGT GTC AAT GTC AAC AAC  1809

Arg Thr Leu Leu Leu Ala Gly Ser Pro Thr Trp Lys Asp Thr Ser Ser Gln Gly His Leu   587
AGG ACT TTG CTC CTG GCT GGA AGC CCG ACC TGG AAG GAC ACC AGT AGT CAG GGC CAC TTG  1869

Phe Arg Thr Arg Asp Glu Lys Gln Ser Pro Gly Arg Val Tyr Gly Tyr Phe Pro Pro Ile   607
TTC CGC ACT CGT GAT GAG AAA CAG AGC CCT GGA CGG GTG TAC GGC TAT TTC CCG CCA ATC  1929

Cys Gln Ser Trp Phe Thr Ile Ser Gly Asp Lys Ala Met Gly Lys Leu Gly Thr Ser Leu   627
TGT CAA AGC TGG TTT ACC ATT TCC GGA GAC AAG GCA ATG GGG AAA CTG GGT ACC TCC CTG  1989

Ser Ser Gly His Val Ile Val Asn Gly Thr Arg Thr Gln Val Leu Leu Val Gly Ala Pro   647
TCT AGT GGC CAC GTG ATG GTG AAC GGG ACC CGG ACC CAA GTG CTG CTG GTG GGG GCC CCG  2049

Thr Gln Asp Val Val Ser Lys Val Ser Phe Leu Thr Met Thr Leu His Gln Gly Gly Ser   667
ACT CAA GAT GTC GTG TCT AAG GTA TCA TTC CTG ACC ATG ACC CTG CAC CAA GGT GGG AGC  2109

Thr Arg Met Tyr Glu Leu Thr Pro Asp Ser Gln Pro Ser Leu Leu Ser Thr Phe Ser Gly   687
ACT CGG ATG TAT GAA CTG ACA CCT GAC TCA CAG CCT TCT CTG CTC AGC ACC TTC AGT GGA  2169

Asn Arg Arg Phe Ser Arg Phe Gly Gly Val Leu His Leu Ser Asp Leu Asp Asn Asp Gly   707
AAC CGC CGC TTC TCC CGA TTT GGT GGC GTT CTG CAC TTG AGT GAC TTG GAT AAT GAT GGC  2229

Leu Asp Glu Ile Ile Val Ala Ala Pro Leu Arg Ile Thr Asp Ala Thr Ala Gly Leu Met   727
TTA GAT GAA ATC ATC GTA GCA GCC CCG CTG AGG ATC ACA GAC GCA ACT GCG GGA CTG ATG  2289

Gly Glu Glu Asp Gly Arg Val Tyr Val Phe Asn Gly Lys Gln Ile Thr Val Gly Asp Val   747
GGG GAA GAG GAT GGC CGT GTT TAT GTG TTT AAT GGC AAA CAG ATC ACC GTG GGT GAC GTG  2349

Thr Gly Lys Cys Lys Ser Trp Val Thr Pro Cys Pro Glu Glu Lys Ala Gln Tyr Val Leu   767
ACA GGC AAA TGC AAA TCA TGG GTA ACT CCG TGT CCA GAA GAA AAG GCC CAA TAT GTA CTA  2409

Ile Ser Pro Glu Ala Gly Ser Arg Phe Gly Ser Ser Val Ile Thr Val Arg Ser Lys Glu   787
ATT TCT CCT GAA GCA GGC TCA AGG TTT GGG AGC TCT GTG ATC ACT GTG AGG TCG AAG GAA  2469

Lys Asn Gln Val Ile Ile Ala Ala Gly Arg Ser Ser Leu Gly Ala Arg Leu Ser Gly Val   807
AAG AAT CAA GTC ATC ATT GCT GCT GGA AGG AGT TCT CTG GGA GCC CGA CTC TCT GGG GTG  2529

Leu His Ile Tyr Arg Leu Gly Gln Asp End                                          816
CTT CAT ATC TAT AGG CTC GGC CAA GAT TAA AGGTTTCACTCCATTTTTC                      2578
```

FIG 6

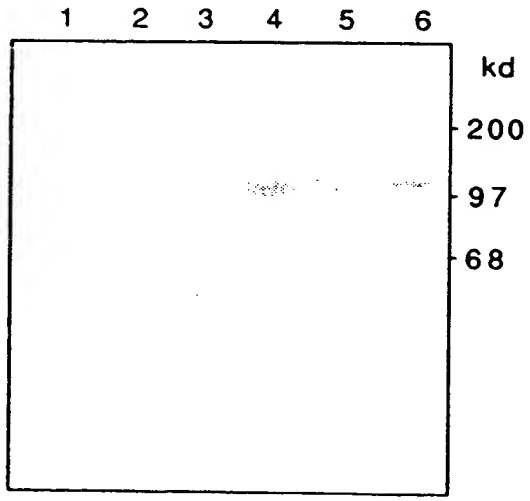


FIG 7

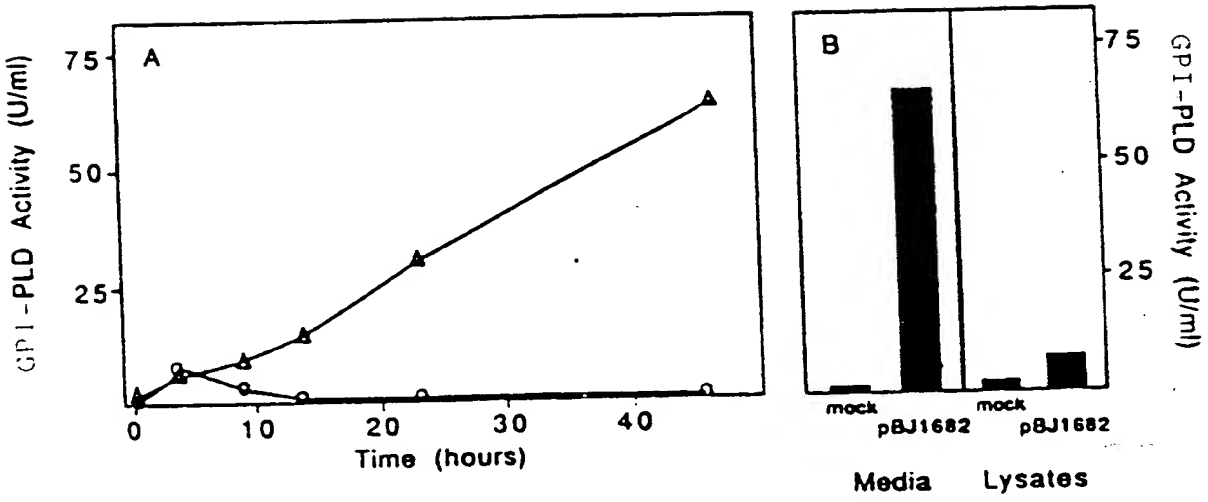

A
GPI-PLD Activity (U/ml)
75
50
25
0
10
20
30
40
Time (hours)
B
75
50
25
GPI-PLD Activity (U/ml)
mock
pBJ1682
mock
pBJ1682
Media
Lysates

FIG 8

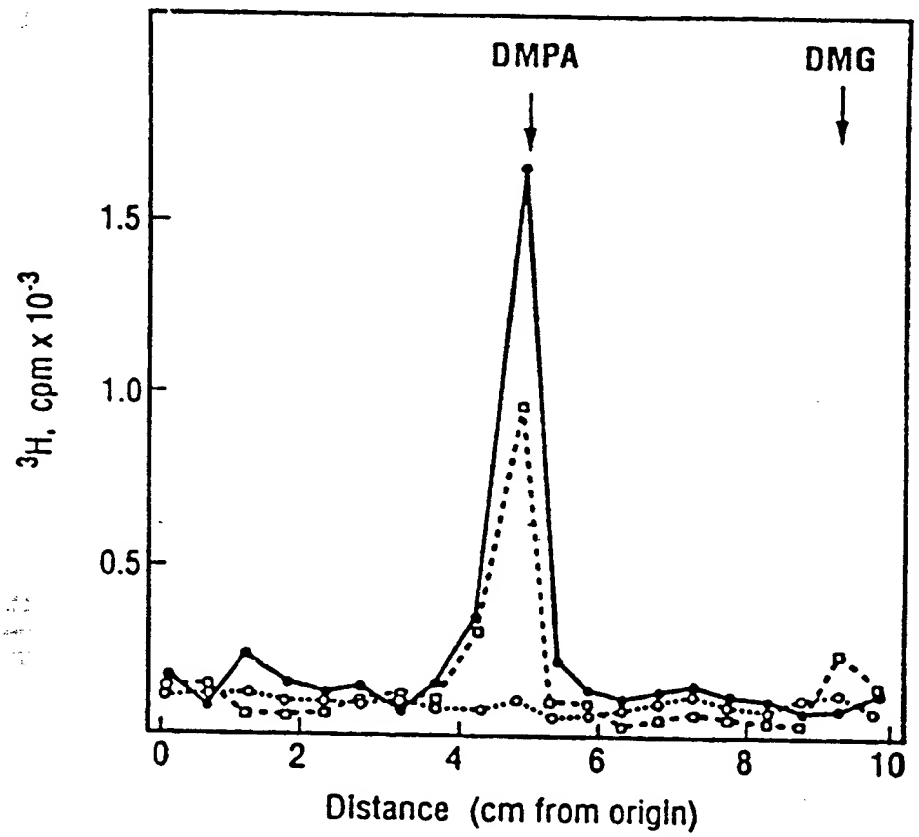


FIG 9

Nucleotide sequence and deduced amino acid sequence of Human Liver GPI-Phospholipase D cDNA

```
                                        1
cgtcattagaggagccggtggggaatgagagcATGTCTGCTTTCAGGTTGTGGCCCGGCC
--------+--------+--------+--------+--------+--------+--------+ 28
gcagtaatctcctcggccacccccttactctcgTACAGACGAAAGTCCAACACCGGGCCGG
                                       -24
                                        M  S  A  F  R  L  W  P  G  L -15

TGCTGATGATCGTGATGGCTTCTCTCTGCCATAGAGGTTCATCGTGTGGCCTTTCAACGC
--------+--------+--------+--------+--------+--------+--------+ 88
ACGACTACTAGCACTACCGAAGAGAGACGGTATCTCCAAGTAGCACACCGGAAAGTTGCG
                                                +1
  L  M  I  V  M  A  S  L  C  H  R  G  S  S  C  G  L  S  T  H +6

ACATAGAAATCGGACACAGAGCTCTGGAGTTTCTTCATCTTCACAATGGGCATGTTAACT
--------+--------+--------+--------+--------+--------+--------+ 148
TGTATCTTTAGCCTGTGTCTCGAGACCTCAAAGAAGTAGAAGTGTTACCCGTACAATTGA

  I  E  I  G  H  R  A  L  E  F  L  H  L  H  N  G  H  V  N  Y 26

ACAAAGAGCTGTTACTAGAACACCAGGATGCATATCAGGCTGGAACCGTGTTTCCTGATT
--------+--------+--------+--------+--------+--------+--------+ 208
TGTTTCTCGACAATGATCTTGTGGTCCTACGTATAGTCCGACCTTGGCACAAAGGACTAA

  K  E  L  L  L  E  H  Q  D  A  Y  Q  A  G  T  V  F  P  D  C 46

GTTTTTACCCTAGCCTCTGCAAAGGAGGAAAATTCCATGATGTGTCTGAGAGCACTCACT
--------+--------+--------+--------+--------+--------+--------+ 268
CAAAAATGGGATCGGAGACGTTTCCTCCTTTTAAGGTACTACACAGACTCTCGTGAGTGA

  F  Y  P  S  L  C  K  G  G  K  F  H  D  V  S  E  S  T  H  W 66

GGACTCCGTTTCTTAACGCAAGCGTTCATTATATCCGAGAGAACTATCCCCTTCCCTGGG
--------+--------+--------+--------+--------+--------+--------+ 328
CCTGAGGCAAAGAATTGCGTTCGCAAGTAATATAGGCTCTCTTGATAGGGGAAGGGACCC

  T  P  F  L  N  A  S  V  H  Y  I  R  E  N  Y  P  L  P  W  E 86

AGAAGGACACAGAGAAACTGGTAGCTTTCTTGTTTGGAATTACTTCTCATATGGTAGCAG
--------+--------+--------+--------+--------+--------+--------+ 388
TCTTCCTGTGTCTCTTTGACCATCGAAAGAACAAACCTTAATGAAGAGTATACCATCGTC

  K  D  T  E  K  L  V  A  F  L  F  G  I  T  S  H  M  V  A  D 106
```

FIG 9 (continued)

```
ATGTCAGCTGGCATAGTCTGGGCATTGAACAAGGATTCCTTAGGACCATGGGAGCTATTG
---------+---------+---------+---------+---------+---------+---------+ 448
TACAGTCGACCGTATCAGACCCGTAACTTGTTCCTAAGGAATCCTGGTACCCTCGATAAC

  V  S  W  H  S  L  G  I  E  Q  G  F  L  R  T  M  G  A  I  D 126

ATTTTCACGGCTCCTATTCTGAGGCTCATTCAGCTGGTGATTTTGGAGGAGATGTGTTGA
---------+---------+---------+---------+---------+---------+---------+ 508
TAAAAGTGCCGAGGATAAGACTCCGAGTAAGTCGACCACTAAAACCTCCTCTACACAACT

  F  H  G  S  Y  S  E  A  H  S  A  G  D  F  G  G  D  V  L  S 146

GCCAGTTTGAATTTAATTTTAATTACCTTGCACGACGCTGGTATGTGCCAGTCAAAGATC
---------+---------+---------+---------+---------+---------+---------+ 568
CGGTCAAACTTAAATTAAAATTAATGGAACGTGCTGCGACCATACACGGTCAGTTTCTAG

  Q  F  E  F  N  F  N  Y  L  A  R  R  W  Y  V  P  V  K  D  L 166

TGCTGGGAATTTATGAGAAACTCTATGGTCGAGAAGTCATCACTGAAAATGTAATTGTTG
---------+---------+---------+---------+---------+---------+---------+ 628
ACGACCCTTAAATACTCTTTGAGATACCAGCTCTTCAGTAGTGACTTTTACATTAACAAC

  L  G  I  Y  E  K  L  Y  G  R  E  V  I  T  E  N  V  I  V  D 186

ATTGTTCACATATCCAGTTCTTAGAAATGTATGGTGAGATGCTAGCTGTTTCCAAGTTAT
---------+---------+---------+---------+---------+---------+---------+ 688
TAACAAGTGTATAGGTCAAGAATCTTTACATACCACTCTACGATCGACAAAGGTTCAATA

  C  S  H  I  Q  F  L  E  M  Y  G  E  M  L  A  V  S  K  L  Y 206

ATCCCTCTTACTCTACAAAGTCCCCGTTTTTGGTGGAACAATTCCAAGAGTATTTTCTTG
---------+---------+---------+---------+---------+---------+---------+ 748
TAGGGAGAATGAGATGTTTCAGGGGCAAAAACCACCTTGTTAAGGTTCTCATAAAAGAAC

  P  S  Y  S  T  K  S  P  F  L  V  E  Q  F  Q  E  Y  F  L  G 226

GAGGACTGGATGATATGGCGTTTTGGTCCACTAATATTTACCATCTAACGAGCTTCATGT
---------+---------+---------+---------+---------+---------+---------+ 808
CTCCTGACCTACTATACCGCAAAACCAGGTGATTATAAATGGTAGATTGCTCGAAGTACA

  G  L  D  D  M  A  F  W  S  T  N  I  Y  H  L  T  S  F  M  L 246

TGGAGAATGGGACCAGTGACTGCAGCCTACCTGAGAACCCTCTGTTCATTGCATGTGGTG
---------+---------+---------+---------+---------+---------+---------+ 868
ACCTCTTACCCTGGTCACTGACGTCGGATGGACTCTTGGGAGACAAGTAACGTACACCAC

  E  N  G  T  S  D  C  S  L  P  E  N  P  L  F  I  A  C  G  G 266

GCCAGCAAAACCACACCCAGGGCTCGAAAATGCAGAAAAATGATTTTCACAGAAATTTGA
---------+---------+---------+---------+---------+---------+---------+ 928
CGGTCGTTTTGGTGTGGGTCCCGAGCTTTTACGTCTTTTTACTAAAAGTGTCTTTAAACT

  Q  Q  N  H  T  Q  G  S  K  M  Q  K  N  D  F  H  R  N  L  T 286
```

FIG 9 (continued)

```
CTTCATCCCTAACTGAAAACATTGACAGGAATATAAACTATACCGAAAGAGGAGTGTTCT
---------+---------+---------+---------+---------+---------+ 988
GAAGTAGGGATTGACTTTTGTAACTGTCCTTATATTTGATATGGCTTTCTCCTCACAAGA

  S  S  L  T  E  N  I  D  R  N  I  N  Y  T  E  R  G  V  F  F 306

TCAGTGTAAATTCCTGGACCCCGGATTCCATGTCCTTTATCTACAAGGCTTTGGAAAGGA
---------+---------+---------+---------+---------+---------+ 1048
AGTCACATTTAAGGACCTGGGGCCTAAGGTACAGGAAATAGATGTTCCGAAACCTTTCCT

  S  V  N  S  W  T  P  D  S  M  S  F  I  Y  K  A  L  E  R  N 326

ACGTAAGGACAATGTTCATAGGTGGCTCTCAGTTGTCACAGAAGCACATCTCTAGCCCCT
---------+---------+---------+---------+---------+---------+ 1108
TGCATTCCTGTTACAAGTATCCACCGAGAGTCAACAGTGTCTTCGTGTAGAGATCGGGGA

  V  R  T  M  F  I  G  G  S  Q  L  S  Q  K  H  I  S  S  P  L 346

TAGCATCTTACTTCTTGTCATTTCCTTATGCAAGGCTTGGCTGGGCAATGACCTCAGCTG
---------+---------+---------+---------+---------+---------+ 1168
ATCGTAGAATGAAGAACAGTAAAGGAATACGTTCCGAACCGACCCGTTACTGGAGTCGAC

  A  S  Y  F  L  S  F  P  Y  A  R  L  G  W  A  M  T  S  A  D 366

ACCTCAACCAGGATGGGTACGGCGACCTCGTGGTGGGCGCACCAGGCTACAGCCGCCCTG
---------+---------+---------+---------+---------+---------+ 1228
TGGAGTTGGTCCTACCCATGCCGCTGGAGCACCACCCGCGTGGTCCGATGTCGGCGGGAC

  L  N  Q  D  G  Y  G  D  L  V  V  G  A  P  G  Y  S  R  P  G 386

GCCGCATCCACATCGGGCGCGTGTACCTCATCTACGGCAATGAACTGGGTCTGCCGCCCG
---------+---------+---------+---------+---------+---------+ 1288
CGGCGTAGGTGTAGCCCGCGCACATGGAGTAGATGCCGTTACTTGACCCAGACGGCGGGC

  R  I  H  I  G  R  V  Y  L  I  Y  G  N  E  L  G  L  P  P  V 406

TTGACCTGGACCTGGACAAGGAGGCCCACGGGATCCTTGAAGGTTTCCAGCCCTCAGGTC
---------+---------+---------+---------+---------+---------+ 1348
AACTGGACCTGGACCTGTTCCTCCGGGTGCCCTAGGAACTTCCAAAGGTCGGGAGTCCAG

  D  L  D  L  D  K  E  A  H  G  I  L  E  G  F  Q  P  S  G  R 426

GGTTTGGCTCGGCCTTGGCTATGTTGGACTTTAACATGGATGGCGTGCCTGACCTGGCCG
---------+---------+---------+---------+---------+---------+ 1408
CCAAACCGAGCCGGAACCGATACAACCTGAAATTGTACCTACCGCACGGACTGGACCGGC

  F  G  S  A  L  A  M  L  D  F  N  M  D  G  V  P  D  L  A  V 446

TGGGAGCTCCCTCGGTGGGCTCTGAGCAGCTCACCTACAAAGGTGCTGTGTATGTCTACT
---------+---------+---------+---------+---------+---------+ 1468
ACCCTCGAGGGAGCCACCCGAGACTCGTCGAGTGGATGTTTCCACGACACATACAGATGA

  G  A  P  S  V  G  S  E  Q  L  T  Y  K  G  A  V  Y  V  Y  F 466
```

FIG 9 (continued)

```
GGTTCCAAACAAGGAAGAATGTCTTCTTCCCCTAACATCACCATCTCTTGCCAGGACA
+---------+---------+---------+---------+---------+---------+ 1528
CCAAGGTTTGTTCCTTCTTACAGAAGAAGGGGATTGTAGTGGTAGAGAACGGTCCTGT

  G  S  K  Q  G  R  M  S  S  S  P  N  I  T  I  S  C  Q  D  I 486

TACTGTAACTTGGGCTGGACTCTCTTGGCTGCAGATGTGAATGGAGACAGTGAGCCCG
+---------+---------+---------+---------+---------+---------+ 1588
ATGACATTGAACCCGACCTGAGAGAACCGACGTCTACACTTACCTCTGTCACTCGGGC

  Y  C  N  L  G  W  T  L  L  A  A  D  V  N  G  D  S  E  P  D 506

CTGGTCATTGGCTCCCCTTTTGCACCAGGTGGAGGGAAGCAGAAGGGAATTGTGGCTG
+---------+---------+---------+---------+---------+---------+ 1648
GACCAGTAACCGAGGGGAAAACGTGGTCCACCTCCCTTCGTCTTCCCTTAACACCGAC

  L  V  I  G  S  P  F  A  P  G  G  G  K  Q  K  G  I  V  A  A 526

TTTTATTCTGGCCCCAGCCTGAGCAACAAAGAGAAACTGAACGTGGAGGCGGCCAACT
+---------+---------+---------+---------+---------+---------+ 1708
AAAATAAGACCGGGGTCGGACTCGTTGTTTCTCTTTGACTTGCACCTCCGCCGGTTGA

  F  Y  S  G  P  S  L  S  N  K  E  K  L  N  V  E  A  A  N  W 546

ACGGTGAGAGGCGAGGAAGACTTTGCCTGGTTTGGATACTCCCTTCACGGTGTCACTG
+---------+---------+---------+---------+---------+---------+ 1768
TGCCACTCTCCGCTCCTTCTGAAACGGACCAAACCTATGAGGGAAGTGCCACAGTGAC

  T  V  R  G  E  E  D  F  A  W  F  G  Y  S  L  H  G  V  T  V 566

GACAACAGAACCTTGCTGCTGGTTGGGAGCCCGACCTGGAAGAATGCCAGCAGGCTGG
+---------+---------+---------+---------+---------+---------+ 1828
CCTGTTGTCTTGGAACGACGACCAACCCTCGGGCTGGACCTTCTTACGGTCGTCCGACC

  D  N  R  T  L  L  L  V  G  S  P  T  W  K  N  A  S  R  L  G 586

CCGTTTGTTACACATCCGAGATGAGAAAAAGAGCCTTGGGAGGGTGTATGGCTACTTCC
+---------+---------+---------+---------+---------+---------+ 1888
GGCAAACAATGTGTAGGCTCTACTCTTTTTCTCGGAACCCTCCCACATACCGATGAAGG

  R  L  L  H  I  R  D  E  K  K  S  L  G  R  V  Y  G  Y  F  P 606

CACCAAACAGCCAAAGCTGGTTTACCATTGTTGGAGACAAGGCAATGGGGAAACTGGGTA
+---------+---------+---------+---------+---------+---------+ 1948
GTGGTTTGTCGGTTTCGACCAAATGGTAACAACCTCTGTTCCGTTACCCCTTTGACCCAT

  P  N  S  Q  S  W  F  T  I  V  G  D  K  A  M  G  K  L  G  T 626

CTTCCCTGTCCAGTGGCCACGTGCTGATGAATGGAACTCTGACCCAGGTGCTGCTGGTGG
+---------+---------+---------+---------+---------+---------+ 2008
GAAGGGACAGGTCACCGGTGCACGACTACTTACCTTGAGACTGGGTCCACGACGACCACC

  S  L  S  S  G  H  V  L  M  N  G  T  L  T  Q  V  L  L  V  G 646
```

FIG 9 (continued)

```
GAGCCCCGACACGTGATGATGTGTCTAAGATGGCATTCCTGACCATGACCCTGCACCAAG
---------+---------+---------+---------+---------+---------+---------+ 2068
CTCGGGGCTGTGCACTACTACACAGATTCTACCGTAAGGACTGGTACTGGGACGTGGTTC

  A  P  T  R  D  D  V  S  K  M  A  F  L  T  M  T  L  H  Q  G 666

GCGGAGCCACTCGGATGTACGCGCTCACATCCGACCTGCAGCCACCGCTGCTCAGCACCT
---------+---------+---------+---------+---------+---------+---------+ 2128
CGCCTCGGTGAGCCTACATGCGCGAGTGTAGGCTGGACGTCGGTGGCGACGAGTCGTGGA

  G  A  T  R  M  Y  A  L  T  S  D  L  Q  P  P  L  L  S  T  F 686

TCAGCGGAGACCGCCGCTTCTCTCGATTTGGTGGCGTTCTGCACTTGAGTGACCTGGATG
---------+---------+---------+---------+---------+---------+---------+ 2188
AGTCGCCTCTGGCGGCGAAGAGAGCTAAACCACCGCAAGACGTGAACTCACTGGACCTAC

  S  G  D  R  R  F  S  R  F  G  G  V  L  H  L  S  D  L  D  D 706

ATGATGGCGTAGATGAAATCATCGTGGCAGCCCCCCTGAGGATAGCAGATGTAACCTCTG
---------+---------+---------+---------+---------+---------+---------+ 2248
TACTACCGCATCTACTTTAGTAGCACCGTCGGGGGGACTCCTATCGTCTACATTGGAGAC

  D  G  V  D  E  I  I  V  A  A  P  L  R  I  A  D  V  T  S  G 726

GGCTGATTGGGGGAGAAGATGGCCGAGTTTATGTATATAATGGCAAAGAGACCACCCTTG
---------+---------+---------+---------+---------+---------+---------+ 2308
CCGACTAACCCCCTCTTCTACCGGCTCAAATACATATATTACCGTTTCTCTGGTGGGAAC

  L  I  G  G  E  D  G  R  V  Y  V  Y  N  G  K  E  T  T  L  G 746

GTGACATGACTGGCAAATGCAAATCGTGGATGACTCCATGTCCAGAAGAAAAGGCCCAAT
---------+---------+---------+---------+---------+---------+---------+ 2368
CACTGTACTGACCGTTTACGTTTAGCACCTACTGAGGTACAGGTCTTCTTTTCCGGGTTA

  D  M  T  G  K  C  K  S  W  M  T  P  C  P  E  E  K  A  Q  Y 766

ATGTATTGATTTCTCCTGAAGCCAGCTCAAGGTTTGGGAGCTCCCTGATCACCGTGAGGT
---------+---------+---------+---------+---------+---------+---------+ 2428
TACATAACTAAAGAGGACTTCGGTCGAGTTCCAAACCCTCGAGGGACTAGTGGCACTCCA

  V  L  I  S  P  E  A  S  S  R  F  G  S  S  L  I  T  V  R  S 786

CCAAGGCAAAGAATCAAGTCGTCATTGCCGCTGGAAGGAGCTCTTTGGGAGCCCGACTCT
---------+---------+---------+---------+---------+---------+---------+ 2488
GGTTCCGTTTCTTAGTTCAGCAGTAACGGCGACCTTCCTCGAGAAACCCTCGGGCTGAGA

  K  A  K  N  Q  V  V  I  A  A  G  R  S  S  L  G  A  R  L  S 806

CCGGGGCACTTCACGTCTATAGCCTTGGCTCAGATTGA
---------+---------+---------+---------  2526
GGCCCCGTGAAGTGCAGATATCGGAACCGAGTCTAACT
```

FIG 10

Alignment of deduced amino acid sequence of Human and Bovine Liver GPI-Phospholipase D

```
          -24                                                        25
Bovine    MSAFRFWSGL LMLL.GFLCP RSSP.CGIST HIEIGHRALE FLHLQDGSIN
Human     MSAFRLWPGL LMIVMASLCH RGSS.CGLST HIEIGHRALE FLHLHNGHVN
          MSAFR.W.GL LM.....LC. R.S..CG.ST HIEIGHRALE FLHL..G..N

          26                                                         75
Bovine    YKELLLRHQD AYQAGSVFPD SFYPSICERG QFHDVSESTH WTPFLNASVH
Human     YKELLLEHQD AYQAGTVFPD CFYPSLCKGG KFHDVSESTH WTPFLNASVH
          YKELLL.HQD AYQAG.VFPD .FYPS.C..G .FHDVSESTH WTPFLNASVH

          76                                                        125
Bovine    YIRKNYPLPW DEDTEKLVAF LFGITSHMVA DVNWHSLGIE QGFLRTMAAI
Human     YIRENYPLPW EKDTEKLVAF LFGITSHMVA DVSWHSLGIE QGFLRTMGAI
          YIR.NYPLPW ..DTEKLVAF LFGITSHMVA DV.WHSLGIE QGFLRTM.AI

          126                                                       175
Bovine    DFHNSYPEAH PAGDFGGDVL SQFEFKFNYL SRHWYVPAED LLGIYRELYG
Human     DFHGSYSEAH SAGDFGGDVL SQFEFNFNYL ARRWYVPVKD LLGIYEKLYG
          DFH.SY.EAH .AGDFGGDVL SQFEF.FNYL .R.WYVP..D LLGIY..LYG

          176                                                       225
Bovine    RIVITKKAIV DCSYLQFLEV YAEMLAISKL YPTYSVKSPF LVEQFQEYFL
Human     REVITENVIV DCSHIQFLEM YGEMLAVSKL YPSYSTKSPF LVEQFQEYFL
          R.VIT...IV DCS..QFLE. Y.EMLA.SKL YP.YS.KSPF LVEQFQEYFL

          226                                                       275
Bovine    GGLEDMAFWS TNIYHLTSYM LKNGTSNCNL PENPLFITCG GQQNNTHGSK
Human     GGLDDMAFWS TNIYHLTSFM LENGTSDCSL PENPLFIACG GQQNHTQGSK
          GGL.DMAFWS TNIYHLTS.M L.NGTS.C.L PENPLFI.CG GQQN.T.GSK

          276                                                       325
Bovine    VQKNGFHKNV TAALTKNIGK HINYTKRGVF FSVDSWTMDS LSFMYKSLER
Human     MQKNDFHRNL TSSLTENIDR NINYTERGVF FSVNSWTPDS MSFIYKALER
          .QKN.FH.N. T..LT.NI.. .INYT.RGVF FSV.SWT.DS .SF.YK.LER

          326                                                       375
Bovine    SIREMFIGSS Q.PLTHVSSP AASYYLSFPY TRLGWAMTSA DLNQDGYGDL
Human     NVRTMFIGGS QLSQKHISSP LASYFLSFPY ARLGWAMTSA DLNQDGYGDL
          ..R.MFIG.S Q....H.SSP .ASY.LSFPY .RLGWAMTSA DLNQDGYGDL

          376                                                       425
Bovine    VVGAPGYSHP GRIHVGRVYL IYGNDLGLPR IDLDLDKEAH GILEGFQPSG
Human     VVGAPGYSRP GRIHIGRVYL IYGNELGLPP VDLDLDKEAH GILEGFQPSG
          VVGAPGYS.P GRIH.GRVYL IYGN.LGLP. .DLDLDKEAH GILEGFQPSG
```

FIG 10 (continued)

```
          426                                                       475
Bovine    RFGSAVAVLD FNVDGVPDLA VGAPSVGSEK LTYTGAVYVY FGSKQGQLSS
Human     RFGSALAMLD FNMDGVPDLA VGAPSVGSEQ LTYKGAVYVY FGSKQGRMSS
          RFGSA.A.LD FN.DGVPDLA VGAPSVGSE. LTY.GAVYVY FGSKQG..SS

          476                                                       525
Bovine    SPNVTISCQD TYCNLGWTLL AADVNGDSEP DLVIGSPFAP GGGKQKGIVA
Human     SPNITISCQD IYCNLGWTLL AADVNGDSEP DLVIGSPFAP GGGKQKGIVA
          SPN.TISCQD .YCNLGWTLL AADVNGDSEP DLVIGSPFAP GGGKQKGIVA

          526                                                       575
Bovine    AFYSGSSYSS REKLNVEAAN WMVKGEEDFA WLGYSLHGVN VNNRTLLLAG
Human     AFYSGPSLSN KEKLNVEAAN WTVRGEEDFA WFGYSLHGVT VDNRTLLLVG
          AFYSG.S.S. .EKLNVEAAN W.V.GEEDFA W.GYSLHGV. V.NRTLLL.G

          576                                                       625
Bovine    SPTWKDTSSQ GHLFRTRDEK QSPGRVYGYF PPICQSWFTI SGDKAMGKLG
Human     SPTWKNASRL GRLLHIRDEK KSLGRVYGYF PPNSQSWFTI VGDKAMGKLG
          SPTWK..S.. G.L...RDEK .S.GRVYGYF PP..QSWFTI .GDKAMGKLG

          626                                                       675
Bovine    TSLSSGHVMV NGTRTQVLLV GAPTQDVVSK VSFLTMTLHQ GGSTRMYELT
Human     TSLSSGHVLM NGTLTQVLLV GAPTRDDVSK MAFLTMTLHQ GGATRMYALT
          TSLSSGHV.. NGT.TQVLLV GAPT.D.VSK ..FLTMTLHQ GG.TRMY.LT

          676                                                       725
Bovine    PDSQPSLLST FSGNRRFSRF GGVLHLSDLD NDGLDEIIVA APLRITDATA
Human     SDLQPPLLST FSGDRRFSRF GGVLHLSDLD DDGVDEIIVA APLRIADVTS
          .D.QP.LLST FSG.RRFSRF GGVLHLSDLD .DG.DEIIVA APLRI.D.T.

          726                                                       775
Bovine    GLMGEEDGRV YVFNGKQITV GDVTGKCKSW VTPCPEEKAQ YVLISPEAGS
Human     GLIGGEDGRV YVYNGKETTL GDMTGKCKSW MTPCPEEKAQ YVLISPEASS
          GL.G.EDGRV YV.NGK..T. GD.TGKCKSW .TPCPEEKAQ YVLISPEA.S

          776                                                  817
Bovine    RFGSSVITVR SKEKNQVIIA AGRSSLGARL SGVLHIYRLG QD*
Human     RFGSSLITVR SKAKNQVVIA AGRSSLGARL SGALHVYSLG SD*
          RFGSS.ITVR SK.KNQV.IA AGRSSLGARL SG.LH.Y.LG .D*
```

FIG 11

The Nucleotide Sequence and Amino Acid Sequence of the Human Pancreatic Glycosyl Phosphatidyl Inositol Specific-Phospholipase D.

```
   1  GACAGTGAACCCGATCTGGTCATCGGCTCCCCTTTTGCACCAGGTGGAGGGAAGCAGAAG   60
   1  AspSerGluProAspLeuValIleGlySerProPheAlaProGlyGlyGlyLysGlnLys   20

  61  GGAATTGTGGCTGCGTTTTATTCTGGCCCCAGCCTGAGCGACAAAGAAAAACTGAACGTG  120
  21  GlyIleValAlaAlaPheTyrSerGlyProSerLeuSerAspLysGluLysLeuAsnVal   40

 121  GAGGCAGCCAACTGGACGGTGAGAGGCGAGGAAGACTTCTCCTGGTTTGGATATTCCCTT  180
  41  GluAlaAlaAsnTrpThrValArgGlyGluGluAspPheSerTrpPheGlyTyrSerLeu   60

 181  CACGGTGTCACTGTGGACAACAGAACCTTGCTGTTGGTTGGGAGCCCGACCTGGAAGAAT  240
  61  HisGlyValThrValAspAsnArgThrLeuLeuLeuValGlySerProThrTrpLysAsn   80

 241  GCCAGCAGGCTGGGCCATTTGTTACACATCCGAGATGAGAAAAAGAGCCTTGGGAGGGTG  300
  81  AlaSerArgLeuGlyHisLeuLeuHisIleArgAspGluLysLysSerLeuGlyArgVal  100

 301  TATGGCTACTTCCCACCAAACGGCCAAAGCTGGTTTACCATTTCTGGAGACAAGGCAATG  360
 101  TyrGlyTyrPheProProAsnGlyGlnSerTrpPheThrIleSerGlyAspLysAlaMet  120

 361  GGGAAACTGGGTACTTCCCTTTCCAGTGGTCACGTACTGATGAATGGGACTCTGAAACAA  420
 121  GlyLysLeuGlyThrSerLeuSerSerGlyHisValLeuMetAsnGlyThrLeuLysGln  140

 421  GTGCTGCTGGTTGGAGCCCCTACGTACGATGACGTGTCTAAGGTGGCATTCCTGACCGTG  480
 141  ValLeuLeuValGlyAlaProThrTyrAspAspValSerLysValAlaPheLeuThrVal  160

 481  ACCCTACACCAAGGCGGAGCCACTCGCGTGTACGCACTCATATCTGACGCGCAGCCTCTG  540
 161  ThrLeuHisGlnGlyGlyAlaThrArgValTyrAlaLeuIleSerAspAlaGlnProLeu  180

 541  CTGCTCAGCACCTTCAGCGGAGACCGCCGCTTCTCCCGATTTGGTGGCGTTCTGCACTTG  600
 181  LeuLeuSerThrPheSerGlyAspArgArgPheSerArgPheGlyGlyValLeuHisLeu  200

 601  AGTGACCTGGATGATGATGGCTTAGATGAAATCATCATGGCAGCCCCCCTGAGGATAGCA  660
 201  SerAspLeuAspAspAspGlyLeuAspGluIleIleMetAlaAlaProLeuArgIleAla  220

 661  GATGTAACCTCTGGACTGATTGGGGGAGAAGACGGCCGAGTATATGTATATAATGGCAAA  720
 221  AspValThrSerGlyLeuIleGlyGlyGluAspGlyArgValTyrValTyrAsnGlyLys  240

 721  GAGACCACCCTTGGTGACATGACTGGCAAATGCAAATCATGGATAACTCCATGTCCAGAA  780
 241  GluThrThrLeuGlyAspMetThrGlyLysCysLysSerTrpIleThrProCysProGlu  260

 781  GAAAAGGCGCAATATGTATTGATTTCTCCTGAAGCCAGCTCAAGGTTTGGGAGCTCCCTC  840
 261  GluLysAlaGlnTyrValLeuIleSerProGluAlaSerSerArgPheGlySerSerLeu  280

 841  ATCACCGTGAGGTCCAAGGCAAAGAACCAAGTCGTCATTGCTGCTGGAAGGAGTTCTTTG  900
 281  IleThrValArgSerLysAlaLysAsnGlnValValIleAlaAlaGlyArgSerSerLeu  300

 901  GGAGCCCGACTCTCCGGGGCACTTCACGTCTATAGCCTTGGCTCAGATTGAAGATTTCAC  960
 301  GlyAlaArgLeuSerGlyAlaLeuHisValTyrSerLeuGlySerAsp              320

 961  TGCATTTCCCCACTCTGCCCACCTCTCTCATGCTGAATCACATCCATGGTGAGCATTTTG 1020

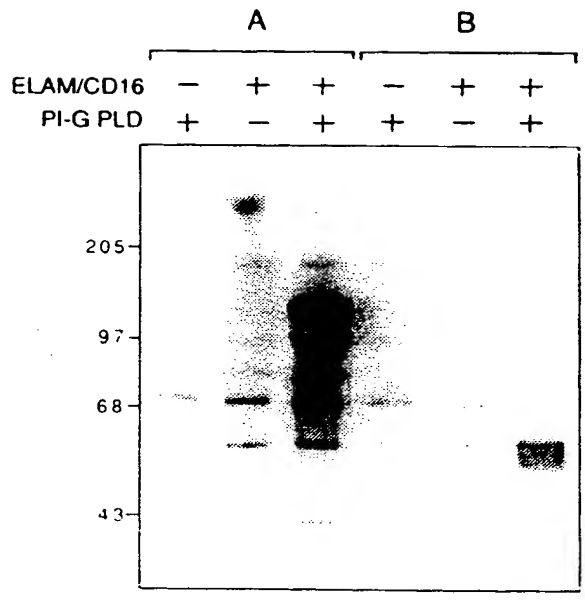
1021  ATGGACAAAGTGGCACATCCAGTGGAGCGGTGGTAGATCCTGATAGACATGGGGCTCCTG 1080

1081  GGAGTAGAGAGACACACTAACAGCCACACCCTCTGGAAATCTGATACAGTAAATATATGA 1140

1141  CTACACCAGAAATATGTGAAATAGCAGACATTCTGCTTACTCATGTCTCCTTCCACAGTT 1200

1201  TACTTCCTCGCTCCCTTTGCATCTAAACCTTTCTTCTTTCCCAACTTATTGCCTGTAGTC 1260
```

FIG 12



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(54) Glycosyl-Phosphatidylinositol-Specific Phospholipase D.

(57) The present invention relates to the protein glycosyl phosphatidylinositol-specificphospholipase D (GPI-PLD) in a substantially pure form, an polynucleotide coding for GPI-PLD, vectors containing the isolated polynucleotide coding for GPI-PLD, and cells transformed by a vector containing the polynucleotide coding for GPI-PLD. Also described is a method for producing a protein which can be secreted from a eukaryotic cell comprising co-transfecting a eukaryotic cell with a gene encoding a glycosyl phosphatidylinositol-anchored protein with glycosyl phospatidylinositol-specific phospholipase D.

EP 0 477 739 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 11 5787

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	THE FASEB JOURNAL, (Abstracts: American Society for Biochemistry and Molecular Biology, The American Association of Immunologists Joint Meeting, New Orleans, Louisiana, 4th - 7th June 1990), vol. 4, no. 7, 26th April 1990, page A1775, abstract no. 474, Bethesda, US; W.-J. FUNG et al.: "Purification of glycosyl phosphatidylinositol-specific phospholipase D by immunoaffinity chromatography" * Abstract * ---	1-4,9-17,22	C 12 N 15/55 C 12 N 9/16 C 12 P 21/02 C 12 P 21/08 A 61 K 39/395
Y	IDEM ---	5-8,18-20,21	
D,Y	PROC. NATL. ACAD. SCI. OF USA, vol. 85, February 1988, pages 980-984, Washington, DC, US; M.G. LOW et al.: "A phospholipase D specific for the phosphatidylinositol anchor of cell-surface proteins is abundant in plasma" * Whole document * ---	5-8,21,22	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N
A	IDEM ---	1-4,9-17	
Y	EP-A-0 319 944 (ZYMOGENETICS INC.)(14-06-1989) * Abstract; page 2, last paragraph - page 3, paragraph 1 * --- -/-	18-20	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	17-09-1992	VAN DER SCHAAL C.A.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
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CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid,

namely claims:

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions,

namely:

see sheet -B-

☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid,

namely claims:

☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims.

namely claims.

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EUROPEAN SEARCH REPORT

Application Number

EP 91 11 5787

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 150, no. 1, 15th January 1988, pages 476-482; M.L. GARDOSO DE ALMEIDA et al.: "Identification of an acid-lipase in human serum which is capable of solubilizing glycophosphatidylinositol-anchored proteins" * Whole document * ---	21	
Y	PROC. NATL. ACAD. SCI. OF USA, vol. 85, no. 23, December 1988, pages 8914-8918, Washington, DC, US; D. HERELD et al.: "cDNA encoding the glycosyl-phosphatidylinositol-specific phospholipase C of trypanosoma brucei" * Whole document * ---	21	
A	IDEM ---	12-17, 22	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
O,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 29, 15th October 1990, pages 17738-17745, (Partially presented at the 81st annual meeting of the Am. Soc. Biochim. and Mol. Biol., 3rd - 7th June 1990), K.-S. HUANG et al.: "Purification and characterization of glycosyl-phosphatidylinositol-specific phospholipase D" * Whole document * ---	1-17,22	
Y	IDEM --- -/-	18-21	

The present search report has been drawn up for all claims

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 11 5787

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	SCIENCE, vol. 252, 19th April 1991, pages 446-448; B.J. SCALLON et al.: "Primary structure and functional activity of a phosphatidylinositol-glycan-specific phospholipase D" * Whole document *	1-17,22	
Y	IDEM	18-21	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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& : member of the same patent family, corresponding document

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LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-20 purified GPI-Phospholipase D, polynucleotide coding for this protein and use of the polynucleotide
2. Claim : 21 use of GPI-PLD for cleaving proteins
3. Claim : 22 antibodies against GPI-PLD